FEMS Microbes, 2024, 5, xtae027
DOI: 10.1093/femsmc/xtae027
Advance access publication date: 9 September 2024
Review Article – Microbes & Disease

Enterococcal-host interactions in the gastrointestinal tract and beyond

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Editor: [Lovleen Joshi]

Abstract

The gastrointestinal tract (GIT) is typically considered the natural niche of enterococci. However, these bacteria also inhabit extraintestinal tissues, where they can disrupt organ physiology and cause life-threatening infections. Here, we discuss how enterococci, primarily *Enterococcus faecalis*, interact with the intestine and other host anatomical locations such as the oral cavity, heart, liver, kidney, and vaginal tract. The metabolic flexibility of these bacteria allows them to quickly adapt to new environments, promoting their persistence in diverse tissues. In transitioning from commensals to pathogens, enterococci must overcome harsh conditions such as nutrient competition, exposure to antimicrobials, and immune pressure. Therefore, enterococci have evolved multiple mechanisms to adhere, colonize, persist, and endure these challenges in the host. This review provides a comprehensive overview of how enterococci interact with diverse host cells and tissues across multiple organ systems, highlighting the key molecular pathways that mediate enterococcal adaptation, persistence, and pathogenic behavior.

Keywords: enterococci; gastrointestinal tract; inter-organ dissemination; enterococcal-host interactions; commensal to pathogen transition; dysbiosis

Enterococci are versatile bacteria that can establish in the upper gastrointestinal tract (GIT), such as the oral cavity, and inhabit the intestinal tract as commensals. They can also colonize other anatomical sites, such as the heart, liver, vaginal, and urinary tracts (Goh et al. 2017, Kao and Kline 2019). Disruption of host-enterococcal homeostasis in many of these host sites can lead to invasive infections and life-threatening diseases (Fisher and Phillips 2009, Agudelo Higuita and Huycke 2014, CDC 2019, Kao and Kline 2019). As such, Enterococci are the second leading cause of nosocomial infections and the primary causative agent of central line-associated bacteremia (CDC 2019, Miller et al. 2020, Weiner-Lastinger et al. 2020). Most infections are caused by *Enterococcus faecalis*, followed by *Enterococcus faecium*, both of which exhibit intrinsic tolerance and acquired resistance to antimicrobials (Agudelo Higuita and Huycke 2014, Weiner-Lastinger et al. 2020). Although *E. faecium* is linked to higher mortality rates due to its strong vancomycin resistance, *E. faecalis* is responsible for a larger number of infections (Kao and Kline 2019). The incidence of *E. faecalis* infections has increased in recent decades due to its persistence in healthcare facilities and abundance in the human gut microbiota (Kao and Kline 2019).

When transitioning from commensals to pathogens, enterococci face challenges such as metabolic alterations, exposure to antimicrobials from host to bacterial origin, competition for nutrients, and immune responses (Kao and Kline 2019). Their malleable genomes, intrinsic resistance to antibiotics, and ability to acquire and disseminate antibiotic resistance enable their adaptation to harsh environments (García-Solache and Rice 2019). Additionally, although not always prevalent in all enterococcal species or strains (see Cariolato et al. 2008, Sava et al. 2010, Kim and Marco 2014, Aung et al. 2023), multiple factors (Table 1) can facilitate their survival, efficient adherence, invasion, and/or immune evasion across organs (Jett et al. 1994, Kayaoglu and Ørstavik 2004, Kao and Kline 2019). Proteins such as Esp (enterococcal surface protein), Ace (collagen-binding protein), and Ebp (Endocarditis- and biofilm-associated pilus protein) play roles in binding to oral (Hubble et al. 2003, Salah et al. 2008, Taglialegna et al. 2020, Spiegelman et al. 2022), urinary (Flores-Mireles et al. 2015, Fiore et al. 2019), vaginal (Alhajjar et al. 2020), and cardiac tissues (Nallapareddy et al. 2000, 2008, 2011a, Singh et al. 2010), while the enterococcal polysaccharide antigen (EPA) helps evade phagocytosis by immune cells (Prajsnar et al. 2013, Smith et al. 2019). Gelatinase E (GelE), aggregation substance (AS), and hyaluronidase also play roles in the colonization and persistence of enterococci in these tissues (Hubble et al. 2003, Goh et al. 2017, Kao and Kline 2019). Gelatinases degrade extracellular matrix components and thus aid bacterial spread, while AS facilitates adhesion and aggregation of enterococci in biofilms (Goh et al. 2017, Ch'ng et al. 2019, Kao and Kline 2019). Hyaluronidase breaks down hyaluronic acid polymers in the tissue extracellular matrix (Rice et al. 2009, Dahiya and Kamal 2013, Alghamdi and Shakir 2020, Asmah 2020), supporting damage and inflammation and promoting enterococcal spread within the gut and other extraintestinal sites (Rice et al. 2009, Kao and Kline 2019, Asmah 2020).

Received 2 May 2024; **revised** 5 August 2024; **accepted** 6 September 2024

Table 1. Factors facilitating enterococcal and host interactions.

Enterococcal factors	Description	Function promoting interactions with host organs
Gelatinase E (*gelE*)	A secreted zinc metalloproteinase, encoded in *gelE*, hydrolyzes collagen and fibrinogen, aiding biofilm formation, tissue degradation, and penetration. It is co-transcribed with *sprE* by the Fsr quorum-sensing system (Qin et al. 2000, Geraldes et al. 2022).	Oral cavity: While highly expressed in biofilms, it also facilitates adherence to dentin and promotes carious cavitation and bone resorption by degrading the dentinal matrix (Zoletti et al. 2011, Elgezawi et al. 2022, Peled et al. 2023). Heart: It mediates degradation of fibrin-rich matrices, thereby facilitating bacterial dissemination from vegetations (Thurlow et al. 2010). Liver: GelE-positive enterococci are enriched in the microbiota of liver cancer patients. The prevalence of GelE in these strains correlates with increased gut permeability and the progression of liver tumors through an unknown mechanism (Iida et al. 2021). GIT: It degrades E-cadherin in intestinal tight junctions, and (with SprE) depletes intestinal collagen, increasing gut permeability. GelE also degrades GLP-1, a hormone regulating gut glucose (Steck et al. 2011, Maharshak et al. 2015, Shogan et al. 2015, LeValley et al. 2020). Urinary tract: It aids fibrinogen degradation, enhancing biofilm formation on implanted catheters (Xu et al. 2017).
Serine protease (SprE)	Protease co-transcribed with *gelE*, which regulates autolysis and extracellular DNA release, critical for enterococcal biofilm formation (Thomas et al. 2008).	Oral cavity: SprE remains active across pH ranges, enabling binding to tooth structures even in alkaline environments. It is also associated with tissue destruction and penetration within the oral cavity (Hubble et al. 2003, Thomas et al. 2008, Halkai et al. 2016). GIT: High collagenase-producing strains of *E. faecalis* have been associated with anastomotic leaks through GelE/SprE-mediated depletion of intestinal collagen, followed by the activation of tissue MMP9, which degrades the host extracellular matrix (Shogan et al. 2015). Urinary tract: SprE, GelE, and host proteases interact with fibrinogen, contributing to CAUTIs (Xu et al. 2017).
Enterococcal surface protein (Esp)	Cell surface proteins present in *E. faecium* and *E. faecalis*, predominantly enriched in clinical isolates. Esp has a role in biofilm formation, possibly through an amyloid-based mechanism (Taglialegna et al. 2020, Geraldes et al. 2022, Spiegelman et al. 2022).	Oral cavity: Esp significantly strengthens biofilms against mechanical or degradative disruptions, enhancing *E. faecalis* cell retention. Hence, Esp unfolding, aggregation, and forming amyloid-like fibers further strengthen biofilms at low pH (Taglialegna et al. 2020, Spiegelman et al. 2022). GIT: Growth on bile acids enhances the hydrophobicity of enterococcal cells and, thus, its surface adherence capabilities (Waar et al. 2002). Urinary tract: Esp facilitates *E. faecalis* adherence to fibrinogen and collagen ligands on bladder cells, promoting biofilm formation. Additionally, Esp is implicated in enterococcal kidney colonization and infection *in vivo* (Shankar et al. 1999, Goh et al. 2017).
Aggregation substance (AS)	AS is a surface-bound glycoprotein that enhances hydrophobicity, facilitating adhesion, and thus promoting mixed-species biofilm formation. Three AS variants have been deeply studied—Asc-10, Asa1, and Asp1—encoded on plasmids pAD1, pCF10, and pPD1, respectively. These variants share >90% identity across most of the protein (Chuang et al. 2009, Geraldes et al. 2022).	Oral cavity: *E. faecalis* AS proteins (Asa1, Asc10, and Asp1) are associated with biofilm formation over dentinal tubes, contributing to persistent endodontic infections (Akbari Aghdam et al. 2017). Heart: Expression of AS proteins (Asc-10, Asa 1, and Asp1) may facilitate the binding of *E. faecalis* to the endothelium or NBTE matrix components, promoting larger infective vegetations (Scheld et al. 1985, Hirt et al. 2000, Chuang et al. 2009, Munita et al. 2012, Goh et al. 2017, Barnes et al. 2021). GIT: AS proteins contribute to enterococcal adhesion to intestinal cells (Isenmann et al. 2000, Sartingen et al. 2000, Wells et al. 2000). Immune cells: AS proteins (especially Asa1 and Asc10) have been linked to immune cell adhesion and phagocytosis survival (Rakita et al. 1999, Vanek et al. 1999, Su¨ßmuth et al. 2000).
Adhesion of collagen (Ace)	A cell surface protein that recognizes MSCRAMMs and binds to the host's extracellular matrix components. It is a homolog of the adhesion protein (Acm) found in *E. faecium* (Geraldes et al. 2022).	Oral tissue: Ace forms a surface channel for binding to dentin, shielding the receptor site from antibodies mediated clearance (Sartingen et al. 2000, Distel et al. 2002, Waar et al. 2002, Hubble et al. 2003). Heart: Ace and Acm are crucial for the initial attachment to cardiac tissue. Ace exhibits high binding affinity NBTE matrix components, including collagen (types I and IV) and laminin. Ace is essential for establishing infective endocarditis (IE) in a rat model (Nallapareddy et al. 2000, 2008, Singh et al. 2010).

Table 1. Continued

Enterococcal factors	Description	Function promoting interactions with host organs
Lipoteichoic acid (LTA)	LTA consists of a polyglycerol phosphate chain bound to the membrane by a glycolipid anchor. This cell surface-associated amphipathic molecules serve as a regulator of autolytic wall enzymes, specifically muramidase's (Reichmann and Grundling 2011).	Oral cavity: LTA has been shown to exhibit high affinity to six human salivary proteins (Baik et al. 2016). Heart: LTA is a factor necessary for exacerbate IE in a rabbit model (Schlievert et al. 1997). GIT: LTA is required for enterococcal adherence to intestinal cells (Fabretti et al. 2006, Theilacker et al. 2009, Sava et al. 2010, Wobser et al. 2014). Urinary tract: Purified LTA significantly inhibited attachment to human bladder cells (Wobser et al. 2014). Immune cells: LTA triggers autophagy in macrophages by inhibiting PI3K/AKT/mTOR and upregulating Beclin 1 (Lin et al. 2018). Other host cells: LTA may inhibit RANKL-induced osteoclast formation via the transcription factor RBP-J (Yang et al. 2016, Wang et al. 2019, Yao et al. 2021). In Osteoblasts: It modulates osteogenic differentiation by enhancing autophagic activity (Liu et al. 2017).
Endocarditis antigen A (EfaA)	Biofilm formation linked factor. It has commonly been associated with IE (Jett et al. 1994, Kayaoglu and Ørstavik 2004, Kao and Kline 2019).	Oral cavity: EfaA is found prevalent in *E. faecalis* strains isolated from dental root surfaces, with their presence correlating with increased biofilm formation *in vitro* (Salah et al. 2008, Akbari Aghdam et al. 2017, Bakhti et al. 2021, Rath et al. 2021). Heart: EfaA is a dominant antigen in serum of patients with infectious endocarditis, which shows homology to a family of adhesins from oral streptococcal strains (Lowe et al. 1995, Singh et al. 1998).
Hyaluronidase (HYL)	Enzyme that degrades hyaluronic acid polymers into disaccharides, providing nutrients and fostering colonization (Dahiya and Kamal 2013, Alghamdi and Shakir 2020).	Oral cavity: Hyl produced by *E. faecalis* can break down the hyaluronic acid in dentin, promoting colonization. Hyl can also facilitate the migration of other oral bacteria from the root canal to periapical lesions (Dahiya and Kamal 2013, Alghamdi and Shakir 2020, Asmah 2020). GIT: Hyl enhances gut colonization by enterococcal species, possibly by modulating the degradation of hyaluronic acid polymers in the gut, which could serve as a nutrient source (Rice et al. 2009).
Cytolysin (Cyl)	Protein encoded by the genes *cylLL* and *cylLS*, presents both bactericidal and cytolytic activity (Geraldes et al. 2022).	Liver: *E. faecalis* Cyl causes hepatocyte damage and exacerbates liver damage in patients with alcoholic hepatitis (Duan et al. 2019). GIT: Cyl expression may facilitate enterococcal penetration of the intestinal epithelium via pore formation and lysis of the target cells (Pham et al. 2014, Huycke et al. 1991).
Endocarditis- and biofilm-associated pili (Ebp)	Pili consist in multimeric fibers composed of pilin subunits that extend as long filaments from the cell surfaces. Components encoded in a gene cluster (*ebpABC* in *E. faecalis* and *empABC* in *E. faecium*) (Goh et al. 2017).	Oral cavity: Ebp is prevalent in *E. faecalis* from dental root surfaces, correlating with increased *in vitro* biofilm formation (Salah et al. 2008, Akbari Aghdam et al. 2017, Bakhti et al. 2021). Heart: Epb mediates adhesion and aggregation to human platelets (Nallapareddy et al. 2011). Urinary tract: it mediates adherence to tissues and biofilm formation in the urogenital tract. It aids colonization of kidney and bladder in experimental *in vivo* models (Sillanpää et al. 2010, Nallapareddy et al. 2011, Flores-Mireles et al. 2015). Vaginal tract: Ebp pili is required for attachment to human vaginal and cervical cells *in vitr o* (Alhajjar et al. 2020).
Enterococcal polysaccharide antigen (EPA)	This glycan consists in a rhamnan polymer backbone (EPA core) that binds to cell wall exposed WTAs (ribitol teichoic acids; EPA decorations). EPA biosynthesis is encoded by two genetic loci (Ramos et al. 2021).	GIT: EPA plays a protective role for enterococci in the GIT, countering the membrane-disrupting effects of bile salts and osmotic stress. It enhances GIT colonization possibly by stabilizing and promoting aggegrate formation. Genes within the core and variable region (*epaX*) of the *epa* loci are essential for efficient migration through intestinal epithelial barriers *in vitro* (Ramos et al. 2019, 2021). Immune cells: EPA polysaccharide has been associated with increased resistance to phagocytosis by macrophages aiding for immune evasion (Teng et al. 2002, Prajsnar et al. 2013).

Abbreviations: gastrointestinal tract (GIT); non-bacterial thrombotic endocarditis (NBTE); and catheter-associated urinary infections (CAUTIs).

The versatility and adaptability of enterococci are evident in their ability to form biofilms on numerous surfaces, utilize diverse nutrients, and persist in various host tissues and environments. Therefore, this review provides a comprehensive overview of enterococcal interactions, particularly *E. faecalis*, with the GIT and other organs. We highlight the complex strategies these bacteria employ to adhere and invade tissues, as well as evade immune defenses across the oral cavity, intestine, and beyond (heart, liver, and kidney).

Enterococci in the oral cavity

The oral cavity is a dynamic environment that constantly changes, especially after food intake (Nagakubo and Kaibori 2023).

These changes create ideal growth conditions for some bacteria, including enterococci, which are often seen as opportunistic members of the oral microbiota (Zaatout 2021, Nagakubo and Kaibori 2023). The origin and persistence of enterococci in the mouth are unclear. They may enter as contaminants from the gut through person-to-person transmission or via contaminated food (Zehnder and Guggenheim 2009, Vidana et al. 2011, Lins et al. 2019). *E. faecalis* is the most frequently isolated enterococcal species in this cavity, followed by *E. faecium* (Komiyama et al. 2016), and its prevalence is linked to its ability to endure in saliva (Souto and Colombo 2008, Wang et al. 2012, Gaeta et al. 2023). However, enterococci are rarely recovered from the mouths of healthy individuals (Sedgley et al. 2004), suggesting that a perturbed oral environment may favor the opportunistic establishment of these bacteria.

Structures such as salivary glands, hard tooth surfaces (enamel, dentin, and cementum), and soft tissues like the pulp, tongue, buccal mucosa, palate, and gingiva constitute the oral cavity (Fig. 1; Zhan 2018). Mechanical and chemical insults, influenced by the oral microbiome, can damage hard surfaces, leading to dental caries, fissures, or trauma (Deo and Deshmukh 2019, Li et al. 2022, Pignatelli et al. 2022). This damage exposes sensitive areas like the pulp tissue and root canal system to pathogen and commensal bacteria colonization (Lamont et al. 2018), which can cause root system or periapical infections (Farges 2009, Bolyachin et al. 2022, Zhu et al. 2022, Sobieszczanski et al. 2023). Enterococci are often implicated in endodontic infections, particularly in failed root canal treatments with chronic apical periodontitis (AP; Elashiry et al. 2023). *E. faecalis* constitutes ~45% of the species isolated in chronic AP cases and is commonly associated with secondary or post-treatment infections (Pinheiro et al. 2003, Deng et al. 2023, Gaeta et al. 2023).

Despite its low prevalence in healthy hosts (Aas et al. 2005), *E. faecalis* can infiltrate the root canal, where its adaptability fosters survival as a single- or mixed-species colonizer (Najafi et al. 2020, Elashiry et al. 2023, Sobieszczanski et al. 2023). Its presence and persistence in the root canal, especially in dentin tubules and lateral canals (Sobieszczanski et al. 2023), can lead to the destruction of the pulp, a connective tissue intricately linked with the periodontium, as well as obstruction of tissue blood supply (Fig. 1). This can instigate prolonged inflammation, resulting in periapical tissue lesions, destruction, and bone resorption, resulting in teeth loss (Marton and Kiss 2000, Love and Jenkinson 2002, Stuart et al. 2006, Komiyama et al. 2016, Elashiry et al. 2023, Sobieszczanski et al. 2023).

Early stages of oral surface colonization by enterococci

At early stages of colonization, bacteria must adhere to the tooth's hard surfaces (Lamont et al. 2018). *Enterococcus faecalis* has a strong affinity for dentine beneath the enamel layer, likely mediated by the adhesin Ace (Table 1 and Fig. 1; Love 2001, Hubble et al. 2003, Halkai et al. 2016). Research by Hubble et al. (2003) underscored Ace's importance in enterococcal adhesion to dentin. Using *in vitro* binding assays, they found that Ace-deficient mutants had reduced adherence to dentin compared with their wild-type OG1RF derived from the human oral isolate *E. faecalis* OG1 (Hubble et al. 2003, Dale et al. 2018). In fact, the C-domain of Ace has been shown to bind to collagen type I (Nallapareddy et al. 2000, Singh et al. 2010, Cohen et al. 2013, Venkateswaran et al. 2022), the primary constituent of dentin, comprising up to 90% of intratubular proteins (Goldberg et al. 2011, Elashiry et al. 2023). Additional studies using a different strain (ATTC33186) revealed an upregulation of the Ace gene transcription under conditions that promote *E. faecalis* interactions with dentin, such as alkaline stresses induced during root canal treatment with calcium hydroxide (Ran et al. 2015a). Other enterococcal factors, such as GelE, the enterococcal serine protease SprE, and Asa1 (Fig. 1 and Table 1), have been implicated in facilitating enterococcal adherence to dental tissues (Sartingen et al. 2000, Distel et al. 2002, Waar et al. 2002, Hubble et al. 2003, Halkai et al. 2016). *E. faecalis* OG1RF deficient in SprE or GelE showed a marked decrease in dentin binding *in vitro* (Hubble et al. 2003, Guneser and Eldeniz 2016), indicating these proteases play key roles in initial interactions with the oral surface, possibly enabling an enterococcal persistent colonization.

Even though the precise role of host factors in the enterococcal oral surface attachment process remains elusive, George and Kishen (2007) observed that starvation enhanced binding to dentin pretreated with saliva *in vitro*, concomitant with an increase in enterococcal cell hydrophobicity. In other oral bacteria, binding to salivary proteins appears to play a pivotal role in surface adherence and invasion (Scannapieco 1994, Baik et al. 2016). Interestingly, purified lipoteichoic acid (LTA) from *E. faecalis* exhibited high affinity to six human salivary proteins (Baik et al. 2016), suggesting a potential connection between these components during enterococcal oral infections. In addition to saliva, serum originating from the alveolar bone and the periodontal ligament seems to enhance enterococcal adherence to oral surfaces by promoting bacterial interaction with collagen type I (Love 2001). Additional research is needed to understand how *E. faecalis* interacts with additional host factors to promote its adhesion to dental surfaces and facilitate its establishment.

Oral biofilms and their role in enterococcal tissue persistence

E. faecalis can form complex multicellular structures, biofilms, that aid its long-term colonization of oral surfaces like dentine (Fig. 1; Duggan and Sedgley 2007, Bulacio Mde et al. 2015). AP is a biofilm-induced disease in both treated and untreated root canals (Jhajharia et al. 2015). Enterococci can form aggregates with various oral microbial species *in vitro*, suggesting their coexistence within oral biofilms *in vivo* (Al-Ahmad et al. 2009). Indeed, *E. faecalis* is highly prevalent in subgingival biofilms from periodontitis patients compared to healthy individuals, with >90% also found in saliva (Souto and Colombo 2008). Takemura et al. (2004) highlighted the capacity of enterococcal strains from root canals to colonize and form thick biofilms on gutta-percha points in the presence of serum, linked to refractory periapical periodontitis. Moreover, microscopic analyses revealed distinct stages in the interaction between *E. faecalis* and dentine. It was proposed that enterococcal cells attached to root canal dentine can induce the dissolution of the dentine's mineral fraction, promoting the formation of a reprecipitated apatite layer within mature biofilms (Kishen et al. 2006). This ability to form calcified biofilms on root canal dentine may contribute to enterococcal persistence after endodontic treatment.

Enterococcal biofilms show increased tolerance to antimicrobials and immune clearance (Conwell et al. 2022), contributing to periodontal treatment failures (Duggan and Sedgley 2007, Jhajharia et al. 2015). Consequently, the long-term survival of *E. faecalis* in the alkaline environment created by calcium hydroxide, a common intracanal treatment, is attributed to its ability to form biofilms and acidify its cytoplasm through proton pumps (Distel et al. 2002, Evans et al. 2002, Ran et al. 2013). Scanning electron

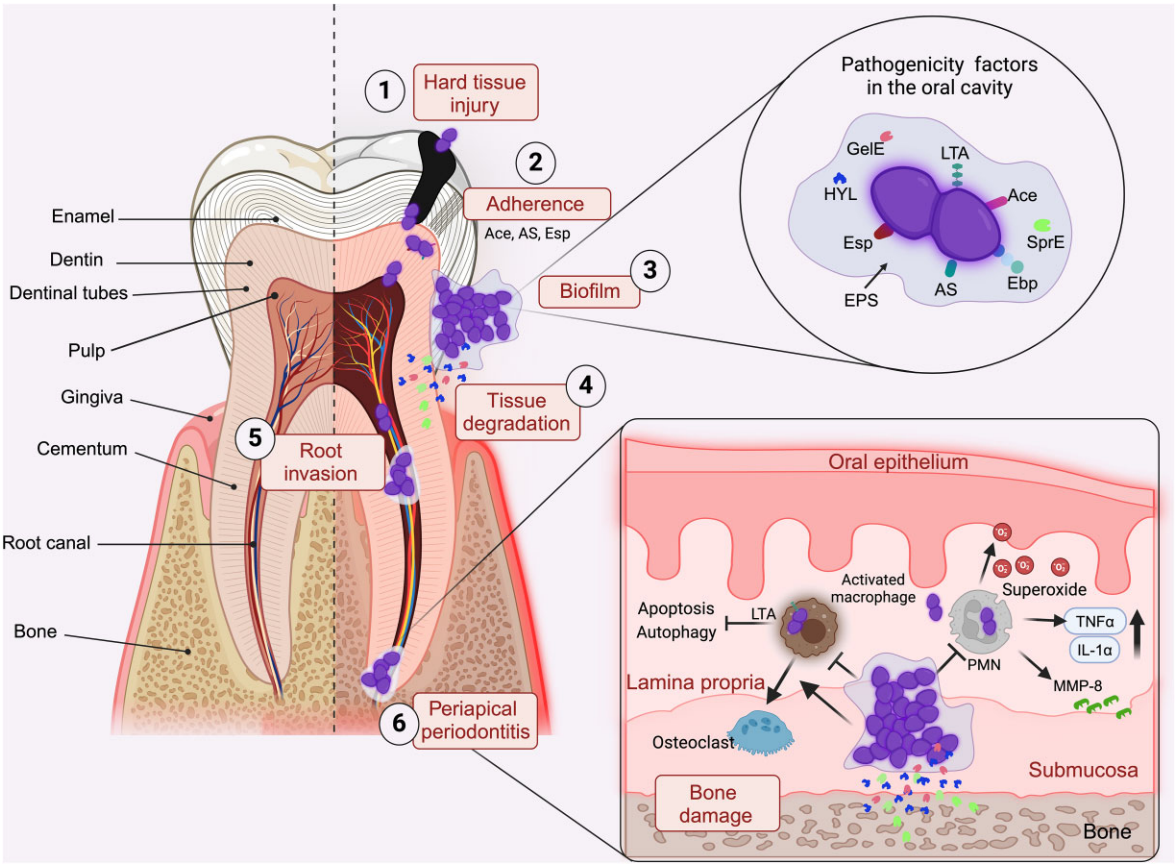

Figure 1. Dynamic interactions of enterococci with oral tissues. The oral cavity includes various structures, with teeth being a prominent component. Each tooth comprises both hard and soft tissues; enamel is the calcified tissue covering the dentin in the crown of the tooth. Dentin, located just beneath the enamel, contains microscopic tubules called dentinal tubules. The cementum is a connective tissue covering the tooth root, attaching to the periodontal ligament. The soft tissues also include the pulp, which contains connective tissue, blood vessels, and nerves. (**1**) Hard tissue injury by mechanical and chemical insults allows enterococcal colonization in sensitive areas like the dentin, pulp tissue, and root canal system. (**2**) *Enterococcus faecalis* adheres to dentin via specific adhesins such as Ace (collagen-binding protein), Esp (enterococcal surface protein), and AS (aggregation substance), which is likely enhanced by salivary proteins. (**3**) Long-term colonization is promoted by biofilm formation on tooth surfaces and within the root canal system, where enterococci are encased by extracellular polymeric substances (EPS) comprised of proteins, fatty acids, and exopolysaccharides. Several enterococcal pathogenicity factors are produced within biofilms, including Esp, which promotes cell retention in this structure, Ebp (Endocarditis- and biofilm-associated pilus protein), and the cell-wall-anchored lipoteichoic acid (LTA). Moreover, enzymes like hyaluronidase (HYL), gelatinase (GelE), and sortase E (SprE) aid the dissolution of dentin's mineral fraction (**4**), promoting calcified biofilm formation and penetration into dentinal tubules that facilitate further invasion and division within the root canals (**5**). The presence of *E. faecalis* in persistent apical periodontitis highlights its capacity to evade immune responses in the periapical region (**6**). Indeed, in this location, Enterococci can inhibit phagocytosis and autophagy in macrophages via LTA while enhancing macrophage differentiation into osteoclasts, resulting in increased bone resorption. Polymorphonuclear leukocytes (PMNs) can migrate into the root canal and respond to *E. faecalis* by producing extracellular superoxide, upregulating proinflammatory factors such as IL-1α and tumor necrosis factor-α (TNF-α), and releasing matrix metalloprotease (MMP-8), which collectively contributes to tissue damage. Enterococcal biofilms increase tolerance to antimicrobials and immune clearance by PMNs and macrophages, further promoting bone degradation. Hence, this sustained infection and inflammation in periapical tissues can lead to bone destruction and tooth loss.

microscopy (SEM) analyses revealed that *E. faecalis* (ATCC4083) on root canals exposed to calcium hydroxide forms biofilms encased within filamentous material networks (Distel et al. 2002, Elashiry et al. 2023), similar to the extracellular polymeric substance (EPS; Fig. 1) found in enterococcal biofilms on other host surfaces (Barnes et al. 2017, Ramos et al. 2019). This EPS likely enhances tissue adherence while shielding bacteria from environmental stressors and the penetration of antimicrobials (Flemming 2016, Jakubovics et al. 2021, Ramos et al. 2021).

While the EPS components from enterococcal oral biofilms *in vivo* remain elusive, Ramirez-Mora and collaborators (Ramirez-Mora et al. 2018) examined the matrix of mono- and dual-species biofilms formed on polystyrene plates by *E. faecalis* isolates from infected root canals. Biochemical analyses revealed that the EPS comprised proteins (chaperones and oxidoreductases), high percentages of saturated and monosaturated fatty acids (mainly palmitic, stearic, and oleic acids), and exopolysaccharides (Ramirez-Mora et al. 2018). Unlike EPS from other enterococcal biofilms (Pazur 1982, Hancock and Gilmore 2002, Ramos et al. 2019, 2021), the polysaccharides in the oral strains' biofilms had high proportions of stachyose, a raffinose family tetrasaccharide (Peterbauer et al. 1999, Ramirez-Mora et al. 2018). Stachyose promotes biofilm formation by *Streptococcus mutans* in mixed cultures with sucrose, contributes to extracellular polysaccharide synthesis in other oral bacteria, and serves as a carbon source for the oral microbiota in periodontitis patients (Song and Jacques 1999, Zhang et al. 2014, Nagasawa et al. 2017). However, its role in the physiology of enterococcal endodontic biofilms and their matrices is still unknown. Changes in pH and nutrients significantly alter the enterococcal EPS composition, affecting bacterial hydropho-

bicity and adhesion (Ran et al. 2013, Chen et al. 2017), highlighting *E. faecalis*' dynamic responses to its environment and potentially aiding its long-term survival in treated root canals.

Several enterococcal factors are proposed to contribute to biofilm formation or stability (Thomas et al. 2008, Ch'ng et al. 2019). Among these, the enterococcal surface protein (Esp; Table 1) was detected by Western blotting in 87.5% of isolates from root canal-treated teeth with post-treatment disease, suggesting a preference for Esp-expressing enterococci in periodontal biofilms (Zoletti et al. 2011). However, another study showed that *E. faecalis* OG1RF lacking the pathogenicity island (PAI) harboring the coding sequence for Esp (*esp*) can still form dense biofilms in a fermenter system (Kristich et al. 2004). Not all *E. faecalis* isolates from various origins (endodontic, plaque/saliva, clinical, or food sources) that form biofilms possess the *esp* gene (Anderson et al. 2015, Seneviratne et al. 2017). Additionally, expressing *esp* from *E. faecalis* in an Esp-negative *E. faecium* strain was not sufficient to promote biofilm development (Laverde Gomez et al. 2011). Although *esp* within a PAI-like structure distinct from that of *E. faecalis* has also been identified in some clinical strains of *E. faecium* (van Schaik et al. 2010), Esp seems not essential for enterococcal biofilm production. However, it was demonstrated the N-terminal region significantly strengthens biofilms against mechanical or degradative disruptions, enhancing *E. faecalis* retention within biofilms (Tendolkar et al. 2004, Tendolkar et al. 2005, Spiegelman et al. 2022). This effect is contingent upon an acidic pH, which induces Esp unfolding, aggregation, and the formation of amyloid-like fibers (Taglialegna et al. 2020, Spiegelman et al. 2022). These amyloid-like structures found abundantly as part of the EPS of other bacterial biofilms, have been shown to exert diverse physiological functions, including promoting interactions with host tissues and immune evasion (Romero and Kolter 2014).

In addition to Esp, a high prevalence of GelE-derive enzymatic activity (39%–75%) was found in enterococci isolated from oral surfaces such as root canals with endodontic treatment failure (Barbosa-Ribeiro et al. 2016, Komiyama et al. 2016). Zoletti et al. (2011) further established that while only 50% of *E. faecalis* strains carrying the *gelE* gene from oral surfaces hydrolyzed gelatin, 70% of isolates from diseased teeth exhibited this enzymatic activity, compared to only 30% of those recovered from healthy patients. They suggested a link between gelatinase production and biofilm formation, with 70% of gelatinase-expressing strains forming robust biofilms. This finding aligns with other studies indicating that enterococci capable of forming biofilms exhibit higher *gelE* expression than biofilm-negative strains (Wang et al. 2011, Zoletti et al. 2011). Kristich et al. (2004) proposed that gelatinase might process signal peptides into mature components or proteolytically activate surface proteins crucial for biofilm development (Fig. 1), such as those involved in EPS secretion. Additionally, genes like Asa1, EfaA (endocarditis antigen A), and EbpR (required for Ebp pilus synthesis; Table 1), associated with biofilm formation, are prevalent in *E. faecalis* from dental root surfaces (Salah et al. 2008, Akbari Aghdam et al. 2017, Bakhti et al. 2021, Rath et al. 2021). These findings highlight the genetic prevalence in root canal enterococci, but further research is warranted to elucidate the precise mechanisms underlying the contributions of these factors to oral biofilm formation and associated diseases.

Dentin invasion and penetration

Reinfection of a treated root canal may occur due to bacterial strains persisting in the tubule system even after canal filling, linked to their ability to penetrate microscopic dentinal tubules extending from the pulp chamber (Fig. 1) to the tooth's outer surface (Love 2002, Gaeta et al. 2023). *In vivo*, infections often start from the pulpal side, with microorganisms migrating through the tubules from the root canal (Peters et al. 2000, Kirsch et al. 2017). Root surface debridement, which removes root cementum, can facilitate bacterial penetration from the periodontal pocket into the tubules, especially in root canal-treated teeth lacking host defenses (Rosen et al. 2020). *E. faecalis* can penetrate dentin *ex vivo* (Ran et al. 2015a, Vatkar et al. 2016, Kirsch et al. 2019, Rosen et al. 2020). Previously, an active process model of dentinal penetration involving a regular rate of migration and multiplication was proposed (Perez et al. 1996). However, Kirsch et al. (2017, 2019) found that non-viable enterococci also penetrate dentinal tubules (~266 µm), while viable bacteria reached deeper (~1002 µm) after 28 days in an *in vitro* tooth model of infection. This suggests that a passive process may also facilitate *E. faecalis* migration, potentially involving synergistic, yet unknown, interactions with oral host tissues. SEM analysis revealed that viable enterococci formed colony-like biofilms at the root canal walls and the entrance of the dentinal tubules after one week, whereas non-viable cells were barely visualized in the same locations and had already migrated into the dentinal tissues (Kirsch et al. 2017). Under glucose starvation and alkaline conditions, *E. faecalis* showed an increased ability to form biofilms on root canals but a decreased capacity to penetrate dentin *in vitro* (Ran et al. 2015b), indicating an inverse relationship between biofilm formation and penetration. Nevertheless, other studies have found that after endodontic surgery, bacterial biofilms can still colonize the root canals and penetrate deep into the dentinal tubules (Rosen et al. 2020).

The expression of factors like gelatinase and hyaluronidase by enterococcal biofilms in root canals may contribute to tissue penetration and damage (Dahiya and Kamal 2013, Alghamdi and Shakir 2020, Elgezawi et al. 2022). Gelatinases contribute to carious lesion cavitation by degrading oral components such as dentinal collagen type I, thereby exposing additional mineralized tissue (Elgezawi et al. 2022). Moreover, the degraded collagen provides nutrients for bacterial growth while compromising the adherence of restorative materials to the infected dentin (Peled et al. 2023). On the other hand, hyaluronidase can break down the hyaluronic acid in dentin into disaccharides, also providing nutrients and promoting enterococcal colonization (Dahiya and Kamal 2013, Alghamdi and Shakir 2020). This process could, in turn, enable tissue destruction during cavity formation (Kayaoglu and Orstavik 2004, Coskun 2019). Additionally, *E. faecalis* hyaluronidase facilitates the migration of other oral bacteria from the root canal to periapical lesions, exacerbating tissue damage and inflammation (Asmah 2020). Further studies are needed to determine the mechanistic role of these enzymes in the migration of these pathogenic bacteria into the root canal system and promoting infections.

Host responses and immunomodulation by enterococci in the oral cavity

The presence of *E. faecalis* in most persistent AP cases suggests it interacts with immune cells in the periapical region to support its survival (Rocas et al. 2004). This interaction begins in infected root canals, where bacterial by-products diffuse into periapical tissues, triggering acute inflammation (Marton and Kiss 2000, Takahama et al. 2018). Sustained inflammation leads to tissue damage and bone resorption, ultimately resulting in tooth loss (Marton and Kiss 2000, Love and Jenkinson 2002). Periapical disease, triggered by bacterial infection, typically starts with chronic inflammation marked by granuloma formation (Marton and Kiss 2000). This in-

flammatory environment involves various immune cells, including polymorphonuclear neutrophils (PMNs; Fig. 1), plasma cells, monocytes, and macrophages, which interact through cell-to-cell contact or secretion of bioactive molecules (Nakamura et al. 2002).

PMNs, as primary responders, rapidly migrate into affected tissues, constituting the frontline defense against bacterial invasion from the root canal (Cassatella et al. 2019). Host cells produce an array of chemoattractants, like interleukin (IL)-8, to recruit PMNs to the infection site (Schroder 1992). Additionally, specific bacterial components, known as microorganism-associated molecular patterns (MAMPs), attract leukocytes from the bloodstream during the inflammatory process (Bloes et al. 2015). *E. faecalis* and *E. faecium* produce pheromone peptides, such as CAM373 and cPD1, which stimulate formyl-peptide receptor 1, inducing the influx of neutrophils (Sannomiya et al. 1990, Bloes et al. 2015). Conversely, sonicated extracts of *E. faecalis* can suppress PMN recruitment by downregulating α4 integrin expression (Lee et al. 2004). Additionally, neutrophils have shown lower extracellular superoxide and phagosomal oxidant production when exposed to *E. faecalis* strains lacking AS (Rakita et al. 1999), suggesting that PMNs' oxidative burst may contribute to tissue damage during enterococcal endodontic infections. *In vitro* experiments suggest PMNs respond to *E. faecalis* by releasing the matrix metalloprotease (MMP-8), a collagenase that may facilitate dentin degradation and, thus, enterococcal dentinal penetration (Visse and Nagase 2003, Ma et al. 2011). Elevated MMP-8 levels have been observed in pulpitis and chronic apical periodontitis cases (Cootauco et al. 1993). Contributing to the progression of dental enterococcal invasion, neutrophils further degrade previously bacterial-demineralized dentin (Gitalis et al. 2019, Peled et al. 2023). Moreover, PMNs also upregulate proinflammatory factors (Fig. 1) such as IL-1α, tumor necrosis factor-α (TNF-α), and cyclooxygenase-2 upon infection with oral enterococcal isolates *in vitro* (Ma et al. 2011), thus adding to the inflammatory response and tissue damage in endodontic infections.

In chronic AP, macrophages play roles in protective responses, lesion development, and inflammation maintenance. Their presence in periradicular inflammatory infiltrate varies from 4% to >50% (Marton and Kiss 2000), but studies suggest a significant early influx in periapical granulomas (Kawashima et al. 1996), influencing interactions with enterococci and AP progression. It was demonstrated that enterococcal root canal isolates activate apoptosis, pyroptosis, and necroptosis in macrophages (Chi et al. 2021), likely through PANoptosis (Jiang et al. 2021, Place et al. 2021). Macrophages infected with these isolates exhibited ultrastructural changes characteristic of apoptosis, pyroptosis, and necroptosis while also showing significant upregulation of three PANoptosome effectors: caspase-3 cleavage, pMLKL, and GSDMD-N proteins (Chi et al. 2021). Although our understanding of *E. faecalis*-induced PANoptosis is limited, this pathway orchestrates programmed cell death to confront infections (Jiang et al. 2021, Place et al. 2021). However, *E. faecalis* can resist phagocyte-mediated killing, delaying adaptive immunity, and surviving inside host immune cells (Gentry-Weeks et al. 1999, Rakita et al. 1999, Wei et al. 2021). This bacterium's intracellular survival has been attributed to interference with macrophage apoptotic signals, inhibiting caspase-3 activation, upregulating AKT, and downregulating the phosphoinositide 3-kinases (PI3K) signaling pathways involved in apoptosis (Zou and Shankar 2014, Chi et al. 2021, Deng et al. 2023). In addition, Lin et al. (2018) showed that *E. faecalis* LTA triggers autophagy in macrophages by inhibiting the PI3K/AKT/mTOR pathway and upregulating Beclin1, potentially also promoting this bacterial survival. In contrast, other studies suggest that intracellular *E. faecalis* inhibits autophagy by evading phagosome acidification and inhibiting LC3-II expression, a protein essential for autophagy activation (Zou and Shankar 2014, Zou and Shankar 2016). Transposon insertion sequencing analysis also revealed that *E. faecalis* OG1RF attenuates mannose and fructose metabolism to escape immune clearance and enhance survival in macrophage cell lines, reducing TNF-α and nitric oxide production (Wei et al. 2021). This dynamic interplay, especially in environments like the oral cavity with fluctuating nutrient sources, could significantly affect the host's cellular responses to infection. Further research is necessary to dissect how nutrient availability impacts macrophage-enterococcal interactions during AP.

Hard tissue destruction in chronic AP stems from the dysregulated functions of osteoclasts and osteoblasts. The migration of osteoclast precursors and subsequent osteoclastogenesis play a crucial role in mineralized bone resorption. Macrophages or monocytes can differentiate into osteoclasts, influencing bone healing in periapical tissues (Pereira et al. 2018). *E. faecalis* may induce macrophage/monocyte differentiation into osteoclasts through various pathways (Fig. 1), including promoting RANKL-dependent osteoclast formation via the p38 and ERK1/2 MAPK pathways, through ephrin ligand B2-Eph receptor B4 bidirectional signaling, and in association with the Janus kinase 2/signal transducer and activator of transcription 3 signaling pathways (Wang et al. 2015, Deng et al. 2016, Wang et al. 2019). Nonetheless, other studies argue that *E. faecalis* LTA may inhibit RANKL-induced osteoclast formation via the transcription factor RBP-J (Yang et al. 2016, Wang et al. 2019, Yao et al. 2021). Cytokines like IL-6, IL-1, and TNF-α also regulate osteoclast differentiation and bone resorption, impacting bone metabolism (Yao et al. 2021). *E. faecalis* carbon metabolism and its AS have been found to stimulate macrophage TNF-α release, suggesting a contribution to bone damage during AP (Kayaoglu and Orstavik 2004, Wei et al. 2021). Osteoblasts, which inhibit bone resorption and promote hard tissue formation, can be affected by enterococcal infections. Multiple *in vitro* studies have shown that *E. faecalis* can inhibit pre-osteoblasts by downregulating transcription factors or altering p38 and ERK1/2 pathways (Park et al. 2015, Wang et al. 2016). Moreover, *E. faecalis* LTA has been shown to stimulate osteogenic differentiation by enhancing autophagic activity (Liu et al. 2017).

Enterococci in the intestine

The lower GIT, comprising the small (duodenum, jejunum, and ileum) and large (ascending, transverse, and sigmoid regions) intestines, digest nutrients through enzyme secretion and absorption via specialized epithelial barriers (Fig. 2; Peterson and Artis 2014, Greenwood-Van Meerveld et al. 2017, Hickey et al. 2023). The small intestine absorbs water, sugars, ions, and amino acids, while the large intestine accumulates fiber, breaks down by-products, and synthesizes/absorbs vitamins, often with the help of gut microbiota (Hickey et al. 2023). Smooth muscle peristalsis and segmentation optimize contact with the gut epithelium, which includes absorptive enterocytes and secretory cells such as enteroendocrine, goblet, and Paneth cells. These cells secrete hormones and antimicrobial peptides and produce mucus to maintain digestive and barrier functions (Peterson and Artis 2014, Greenwood-Van Meerveld et al. 2017). While common throughout the intestinal system, these cell types exhibit location preferences. For instance, Paneth cells are primarily found in the small intestine, and enteroendocrine L cells are predominantly located in the ileum and large intestine (Bowcutt et al. 2014, Hickey et

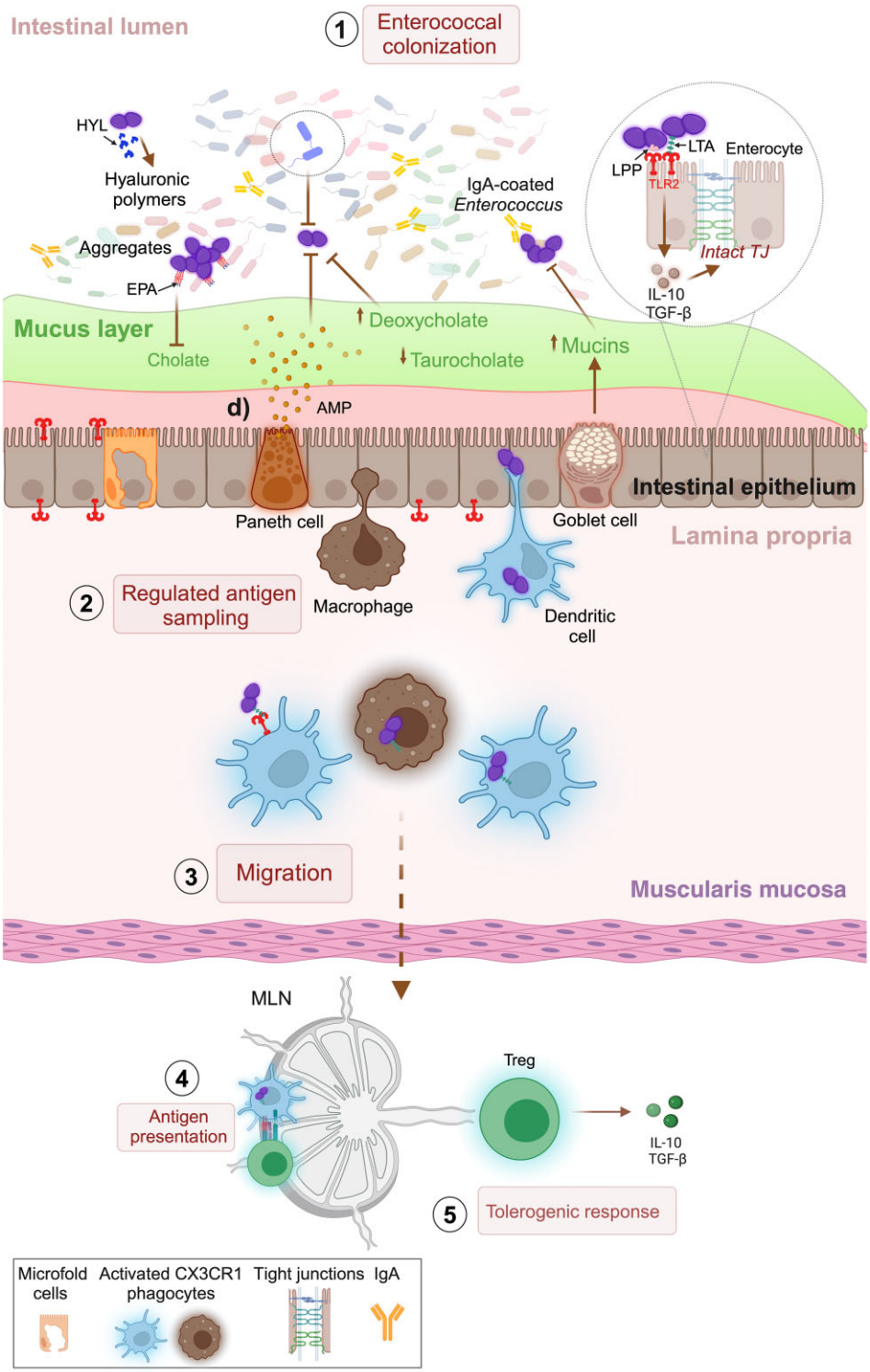

Figure 2. Homeostatic interactions between enterococci and the intestine. Under eubiotic conditions, the long-term colonization (**1**) of enterococci in the gut lumen may be facilitated by multiple processes: the ability to utilize various gut nutrients, such as hyaluronic acid polymers, via hyaluronidases (HYL); the capacity to form biofilms/aggregates in the intestinal mucus layer, counteracting intestinal peristalsis; and the expression of EPA (enterococcal polysaccharide antigen) in the enterococcal cell wall that helps protect bacterial cells against bile acids like cholate. Transient expansion of enterococci in the intestine is limited by several factors, including the secretion of antimicrobial peptides (AMP) by Paneth cells, competition for nutrients with other commensal bacteria, elevated levels of deoxycholate bile acid, and active mucus secretion by Goblet cells. Enterococci on the luminal side can also be coated by IgA secreted by specialized gut plasma cells, preventing their binding to the mucus layer. If reaching the intestinal epithelium, *E. faecalis* lipoteichoic acid (LTA) and/or lipoproteins (LPP) may be recognized by Toll-like receptor (TLR)-2, which can trigger the production of anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) and interleukin (IL)-10, while maintaining tight junction integrity between enterocytes. (**2**) Below the intestinal epithelium, lamina propria dendritic cells and macrophages constantly sample the gut lumen and phagocytose enterococci via TLR2 expressed on these myeloid cells. (**3**) Dendritic cells then migrate to the mesenteric lymph node (MLN), where they present enterococcal antigens to naïve T cells (**4**). This process can lead to the generation of regulatory T cells (Treg), which produce IL-10 and TGF-β, and thus orchestrate tolerance to these gut commensal bacteria (**5**).

al. 2023). Recent studies have shown that the human intestine's unique cell composition is organized into various niches of both epithelial and immune cells. These regions in the intestinal crypts are co-enriched with specific cells: an adaptive immune area at the crypt base, a plasma-cell area in the middle mucosa, and an innate immune zone at the top (Hickey et al. 2023). The mucus barrier differs between the small and large intestines, being approximately four times thicker in the large intestine than in the duodenum and jejunum, and consisting of a more abundant "firm" layer, i.e. difficult to dislodge and considered devoid of bacteria (Johansson et al. 2008, Bowcutt et al. 2014). In contrast, the small intestine mostly contains a soluble mucus gel layer, i.e. not attached to the epithelium and is penetrable by bacteria (Shan et al. 2013, Bowcutt et al. 2014).

The intestinal epithelium's integrity is supported by tight junctions, desmosomes, and adherens junctions, ensuring a robust mucosal immune response alongside the lamina propria's immune cells (Turner 2009, Suzuki 2020). Moreover, specialized plasma cells in the lamina propria produce dimeric IgA that binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral side of intestinal epithelial cells (IECs). Upon binding the pIgR, IgA is transported to the apical surface and released as secretory IgA (sIgA) into the intestinal lumen (Corthésy 2013). sIgA further supports barrier protection against pathogens and promotes symbiosis among commensal bacteria. For more detailed descriptions of this anatomical site and its role in homeostasis, see Kim and Ho (2010), Gallo and Hooper (2012), Peterson and Artis (2014), Greenwood-Van Meerveld et al. (2017), and Suzuki (2020). For more information on intestinal heterogeneity and cellular complexity, see Bowcutt et al. (2014) and Hickey et al. (2023).

Enterococci, part of the GIT microbiota, are primarily found in the jejunum, ileum, cecum, rectum, and colon (Hayashi et al. 2005, Lebreton et al. 2014, 2017, de Almeida et al. 2018, Banla et al. 2019). In healthy humans, enterococci represent only a small fraction (up to 1%; Fig. 2) of the adult intestinal microbiota (Lebreton et al. 2014). Among these, *E. faecalis* and *E. faecium* are the most prevalent species within fecal content (Dubin and Pamer 2014). *E. faecalis* is considered the first colonizer in newborns, having a major impact on intestinal immune development (Fanaro et al. 2003). As commensals, enterococci play a crucial protective role in modulating colonic homeostasis by regulating intestinal pH, producing vitamins, and metabolizing nutrients, while impacting multiple inflammatory responses (de Almeida et al. 2018, Daca and Jarzembowski 2024). Given the differences between the small and large intestines composition, it would be worth understanding how these variations affect the colonization and persistence of enterococci.

Mechanisms of enterococcal tolerance in the intestine

The maintenance of tolerance towards commensal gut microbes, like enterococci, relies on mechanisms that minimize immune cell exposure in the lamina propria to luminal antigens (Peterson and Artis 2014, Burgueno and Abreu 2020, Daca and Jarzembowski 2024). Activation of pattern recognition receptors (PRRs) detecting MAMPs is critical for inducing this tolerance (Peterson and Artis 2014, Burgueno and Abreu 2020). Multiple PRR families, including Toll-like receptors (TLRs), provide pathways to recognize microbial ligands or endogenous signals associated with pathogenesis. IECs express PRRs, functioning as dynamic sensors of the microbial intestinal environment and directing mucosal immune cell responses (Peterson and Artis 2014, Burgueno and Abreu 2020, Daca and Jarzembowski 2024). *E. faecalis* MAMPs, such as LTA and/or lipoproteins, are recognized by TLR2, triggering tolerogenic mechanisms such as the production of anti-inflammatory cytokines (transforming growth factor β, TGF-β, and IL-10) while maintaining tight junction integrity between enterocytes (Fig. 2; Castro et al. 2016, Daca and Jarzembowski 2024). TLR9 detects CpG-rich bacterial DNA, and the cGAS-STING pathway recognizes double-stranded cytosolic DNA (Ewaschuk et al. 2007, Liu et al. 2021). Thus, extracellular DNA present in *E. faecalis* biofilms may activate TLR9 or/and the cGAS-STING pathway on the basolateral surface, potentially modulating the inflammatory response (Ewaschuk et al. 2007, Liu et al. 2021, Daca and Jarzembowski 2024). Notably, TLR expression is downregulated on the apical surfaces of epithelial cells where commensal bacteria reside, but highly upregulated on the basolateral side (Chistiakov et al. 2014). This mechanism ensures only bacteria crossing the epithelial layer are recognized as "foreign." Mice defective in TLR2, TLR4, or the TLR signaling adaptor Myd88 exhibit impaired responses to commensal bacteria and compromised epithelial barrier integrity (Kelly et al. 2005, Bhinder et al. 2014), highlighting the importance of TLR signaling in maintaining commensalism and protection against pathogenic enteric bacteria.

Enterococcal factors facilitating intestinal colonization

Enterococci employ various strategies to thrive in the competitive GIT environment, such as proton extrusion and the regulatory function of two-component systems like EtaRS for gut acid tolerance (Suzuki et al. 1993, Teng et al. 2002, Le Breton et al. 2003, Fiore et al. 2019). To ensure long-term colonization, enterococci must also endure host-secreted antimicrobials and bile acids in the intestine (Ridlon et al. 2014). *E. faecalis* relies on the signaling protein IreK, which is crucial for cell envelope integrity, and resistance to cholate (a cholesterol-derived bile acid) and lysozyme encoded in its core genome. The absence of functional IreK leads to reduced cecum persistence and colonization in mice due to the loss of antimicrobial resistance and cell envelope integrity (Kristich et al. 2007**,** Banla et al. 2018).

Peristalsis, the involuntary muscle contractions and relaxation that propel luminal content, rapidly reduces bacterial density in the intestine (Cremer et al. 2016, Patel and Thavamani 2024). Gut commensals may counteract peristalsis by forming biofilms (Fig. 2) in the intestinal mucus layer, enhancing their persistence in the gut, as mucus turnover is slower than the peristalsis-driven transit time (Sonnenburg et al. 2004). *E. faecalis* biofilm-like microcolonies have been observed throughout the intestine of germ-free mice, indicating biofilm formation at the base of the inner mucus layer (Barnes et al. 2017). Consistent with the previous study, a vancomycin-resistant strain of *E. faecium* showed increased aggregate formation in the cecum of antibiotic-treated mice supplemented with lithocholic acid, suggesting a role for bile acids in biofilm formation. Mutants locked in the non-aggregative state showed deficient colonization and persistence (McKenney et al. 2019). Nevertheless, enterococcal biofilms may serve as reservoirs for consistent colonization of the gastrointestinal lumen, although their persistence within mature microbiota remains unexplored. Enterococcal factors such as EbrB (encoding an AraC family transcriptional regulator), bop locus (biofilm on plastic; locus containing putative maltose metabolism genes), or sortase A (SrtA) (a membrane-associated enzyme mediating anchoring of surface proteins to the enterococcal cell wall) have shown to be important for biofilm development *in vitro* as well as for enhancing

GIT colonization (Creti et al. 2006, Top et al. 2013, Banla et al. 2019). In addition, mutations altering the structure of the enterococcal polysaccharide antigen (EPA), a rhamnose-containing polysaccharide (Guerardel et al. 2020), have demonstrated changes in biofilm formation *in vitro* (Ramos et al. 2021). These mutations also resulted in increased susceptibility to the bile acid cholate, deficient intestinal colonization in a natural colonization model, reduced population expansion in antibiotic-induced dysbiotic mice, and inefficient transmission to juvenile mice following birth (Rigottier-Gois et al. 2015, Chatterjee et al. 2019). Hence, demonstrating the critical role of EPA in enterococcal gut colonization.

Nutritional adaptation promotes the establishment and persistence of enterococci in the GIT, as they can acquire and metabolize various nutrients to outcompete other gut microbes (Banla et al. 2019, Dubin et al. 2014). When colonizing the GIT of germ-free mice, *E. faecalis* has been shown to prioritize expressing genes for nutrient acquisition (like phosphotransferase systems, PTS) and energy metabolism over virulence factors such as GelE or SprE (Lindenstrauss et al. 2014). This suggests that in a less competitive environment, like the gut of a gnotobiotic mouse, enterococcal nutrient acquisition prevails to ensure establishment. Similarly, *E. faecium* lacking *ptsD* encoding a putative PTS shows defects in GIT colonization in mice (Zhang et al. 2013). *E. faecalis'* metabolic plasticity was also evidenced when a mutant deficient in metabolizing ethanolamine (an abundant nutrient in the digestive tract) outcompeted the wild-type strain, thus colonizing the mouse intestinal lumen more efficiently (Kaval et al. 2018). In addition, endogenous plasmids can influence GIT colonization fitness. For instance, Rice et al. (2009) found that acquiring a plasmid containing a hyaluronidase gene enhances GIT colonization by *E. faecium* in an antibiotic-treated mouse model. This trait, which is transferable to other enterococcal strains (Rice et al. 2009), may confer the ability to degrade hyaluronic acid polymers in the gut, using them as a nutrient source. In general, enterococci utilize various carbon sources in the gut (Fig. 2), including non-absorbed sugars (such as lactose and mannose), polymers, and mucins, aiding in their colonization (Chassard et al. 2010, Ramsey et al. 2014, Stein-Thoeringer et al. 2019). Given the differences in composition between the small and large intestines (Bowcutt et al. 2014, Hickey et al. 2023), it is worthwhile to understand how variations, such as nutrient availability, affect the colonization and persistence of enterococci.

Dysbiosis causes enterococcal dominance in the intestine

Diet, chemotherapy, and antibiotic administration can disrupt intestinal homeostasis (eubiosis; Fig. 2), consequently leading to "dysbiosis" (Zeissig and Blumberg 2014, Biedermann and Rogler 2015, Iebba et al. 2016). Antibiotics can induce dysbiosis by creating a less competitive environment susceptible to overgrowth and dominance of pathobionts like *E. faecalis* (Fig. 3) and *E. faecium* (Zeissig and Blumberg 2014, Francino 2015, Chakraborty et al. 2018, Archambaud et al. 2019, Krawczyk et al. 2021). Repoila and collaborators proposed that during eubiosis the abundance of deoxycholate bile acid effectively controls the growth of *E. faecalis* (Repoila et al. 2022). However, upon dysbiosis, the bile acid composition shifts to higher levels of taurocholate (a conjugated bile acid), which the bacterium tolerates, promoting its proliferation. Their *in vitro* studies revealed that deoxycholate, but not taurocholate, inhibits *E. faecalis* growth, affecting the expression of essential genes, including ribosomal proteins (Repoila et al. 2022). Other research has further shown a negative correlation between deoxycholate and *E. faecalis* population in the gut of humans and mice (Iida et al. 2021), suggesting that *E. faecalis* may lack molecular mechanisms to tolerate deoxycholate.

In mice undergoing allogeneic hematopoietic cell transplantation, there was a notable expansion of enterococcal populations in the GIT, which exacerbated the severity of graft-versus-host disease in gnotobiotic models (Stein-Thoeringer et al. 2019). This overgrowth depended on lactose availability, as reducing dietary lactose limited enterococcal expansion and lessened the disease severity in mice (Stein-Thoeringer et al. 2019). Similarly, patients with allogeneic hematopoietic cell transplantation who are lactose-non-absorbers exhibited poor clearance of *Enterococcus* following antibiotic treatment (Stein-Thoeringer et al. 2019). These findings underscore lactose as a critical nutrient that promotes the overgrowth of commensal bacteria, thereby exacerbating both intestinal and systemic inflammatory diseases (Stein-Thoeringer et al. 2019). Enterococcal overgrowth can result from directly inhibiting other intestinal microbes via antimicrobial secretion. *E. faecalis* produces various heat-stable peptide bacteriocins, often encoded on conjugative plasmids, aiding its competition (Nes Ingolf et al. 2007). Indeed, enterococci carrying the plasmid pD1, which encodes a bacteriocin synthesis operon, show enhanced GIT colonization and survival in mice, providing a fitness advantage by displacing preexisting enterococcal populations (Kommineni et al. 2015, 2016). Besides displacing competitors through antimicrobials, enterococci's plastic genome and ability to acquire external genetic material aid their gut proliferation by conferring new fitness traits, such as elevated antibiotic resistance, phage infection endurance, and better metabolic adaptability to new nutrient sources (Banla et al. 2019, García-Solache and Rice 2019).

Enterococcal dominance promotes their exit from the GIT

When enterococcal proliferation in the gut lumen reaches a threshold, it can lead to the breach of the intestinal barrier in hosts with disrupted gut homeostasis, a process known as intestinal translocation (Fig. 3). This allows enterococci to exit the GIT, access the bloodstream, and disseminate to other organs (Archambaud et al. 2019, Kao and Kline 2019, Fine et al. 2020). Experimental mouse models have demonstrated that enterococcal translocation occurs following gut barrier disruption from antibiotic-induced dysbiosis, coinfections, inflammation, injury, alcohol usage, radiation, and decreased gastric acid secretion (Miyazaki et al. 2001, Krueger et al. 2004, Shigematsu et al. 2009, Kobayashi et al. 2012, Heimesaat et al. 2014, Wang et al. 2014, Caballero et al. 2015, Llorente et al. 2017, Soares et al. 2017, Fine et al. 2020). Human studies show that domination of the GIT by vancomycin-resistant enterococci precedes bloodstream infections (Ubeda et al. 2010, Taur et al. 2012, Freedberg et al. 2018). Translocating enterococci have been implicated in causing autoimmunity in genetically predisposed hosts (Manfredo Vieira et al. 2018), and enterococcal-specific DNA was detected in liver biopsies of patients with autoimmune disease (Manfredo Vieira et al. 2018). Furthermore, increased amounts of gut-derived, *E. faecalis*-specific circulating DNA have been found in plasma samples from patients with Crohn's disease and ulcerative colitis, compared with individuals without active intestinal disease (Manfredo Vieira et al. 2018).

Although gut commensals may translocate the intestinal epithelium at lower densities during eubiosis, they are intercepted and eliminated by phagocytes before reaching the bloodstream.

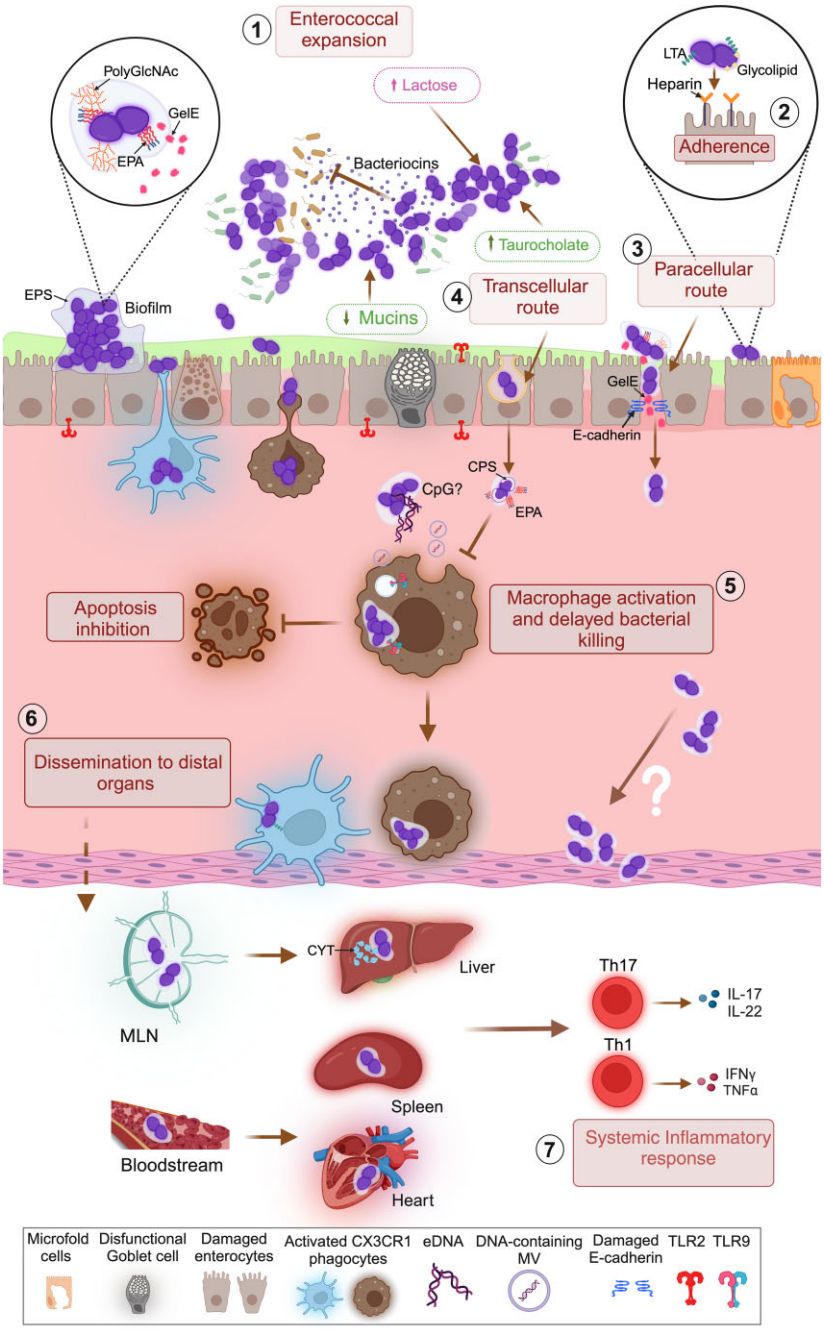

Figure 3. Dysbiosis triggers enterococcal egress from the intestine. Disruption of intestinal homeostasis (dysbiosis) can lead to enterococcal overgrowth/dominance. (**1**) This expansion may be promoted by biofilm formation, where the extracellular polymeric substance (EPS), partly formed by poly *N*-acetylglucosamine (polyGlcNAc)-containing polymers, and the enterococcal polysaccharide antigen (EPA) enhance adherence to surfaces and resistance to antimicrobials and immune responses. Bacteriocin secretion and the ability to metabolize diverse carbon sources (lactose) may also provide a fitness advantage to *E. faecalis*. During dysbiosis, taurocholate levels increase (bile acid), which enterococci can tolerate. Reduced production of antimicrobial peptides, IgA, and mucus further allows *E. faecalis* to adhere to the epithelial layer. (**2**) Moreover, bacterial glycolipids and lipoteichoic acid (LTA), as well as colonic heparin/heparan sulfate receptors, may facilitate enterococcal attachment to epithelial cells. These conditions compromise the epithelial barrier, promoting enterococcal egress from the intestinal lumen (gut translocation) via two routes: paracellularly (**3**), where enterococcal cells adhered to the epithelial layer release gelatinases (GelE) that damage tight junction E-cadherin, allowing bacterial migration between intestinal epithelial cells, and transcellularly (**4**), where *E. faecalis* might pass across the barrier via direct endocytosis by enterocytes. (**5**) Translocated enterococci can be engulfed by lamina propria phagocytes, including CX3CR1 macrophages and dendritic cells. Enterococcal membrane vesicles (MV) containing DNA or extracellular DNA (eDNA) present in biofilms, which may contain CpG motifs, could be recognized by Toll-like receptor (TLR)-9 in lamina propria macrophages. Once phagocytosed, *E. faecalis* can persist/proliferate inside macrophages, especially when taken up as aggregates that inhibit apoptosis in these myeloid cells. *Enterococcus faecalis* can also inhibit phagocytosis through the expression of factors such as EPA and capsule (CPS), which may encase bacterial factors recognized by phagocytes. Enterococcal persistence within phagocytes leads to their activation and production of pro-inflammatory cytokines, aiding their dissemination (**6**) to mesenteric lymph nodes (MLN) and/or the bloodstream, facilitating spread to distal organs such as the liver, spleen, and heart. It is unknown whether extracellular *E. faecalis* can also egress the lamina propria or whether it needs phagocyte activity. Once in distal organs, such as the liver, exotoxins like cytolysin (CYT) may promote disease progression by lysing hepatocytes. (**7**) Enterococci can also trigger a systemic inflammatory response characterized by Th1 and Th17 cell polarization and the production of pro-inflammatory cytokines, such as interleukin (IL)-17, IL-22, interferon-gamma (IFNγ), and tumor necrosis factor-α (TNF-α).

The gut-associated lymphoid tissue (GALT), including Peyer's patches, plays a crucial role in controlling microbial translocation (Jung et al. 2010). Specialized enterocytes called microfold (M) cells, along with macrophages and dendritic cells (DCs), sample luminal contents, allowing some bacteria to bypass the epithelial barrier (Fig. 3). Commensals can be captured by M cells, transported to Peyer's patches, and carried by DCs to mesenteric lymph nodes (MLNs) to initiate IgA responses, and T cell tolerance (Macpherson and Uhr 2004, Jung et al. 2010). Moreover, it has been observed that the gut epithelium of germ-free mice can activate an autophagy pathway, requiring epithelial cell-intrinsic MyD88 signaling, in response to invading commensals like *E. faecalis* or enteric pathogens. Mice with an epithelial cell-specific deletion of a critical autophagy factor (*Atg5*) show increased dissemination to extraintestinal sites, highlighting the importance of this epithelial cell-autonomous mechanism in limiting bacterial spread beyond the intestine (Benjamin et al. 2013). Hence, immune malfunction can promote microbial dissemination to extraintestinal sites. Further studies are essential to elucidate how enterococci affect the function and composition of GALT and other mucosal immune defenses, particularly in the context of dysbiosis and inflammation.

The interaction between the gut microbiota and the host influences intestinal barrier permeability. For example, *E. faecalis* induces intestinal inflammatory responses in IL-10-deficient mouse models of colitis (Kim et al. 2005), potentially compromising barrier function (Steck et al. 2011). Bacterial translocation occurs through two primary mechanisms: paracellular migration between intestinal epithelial cells (IECs) via disruption in tight junctions and transcellular transport across IECs involving apical and basolateral membranes (Balzan et al. 2007). *E. faecalis* has been observed between adjacent IECs and within enterocytes in antibiotic-treated mice, suggesting both paracellular and transcellular pathways for bacterial egress (Ubeda et al. 2010, Peng et al. 2014, Archambaud et al. 2019). However, whether *E. faecalis* or other enterococcal species use both or either route remains unclear. Furthermore, recent work suggests differences in gut translocation capabilities between *E. faecalis* and *E. faecium* (Hendrickx et al. 2015). Antibiotic-driven dysbiosis that promotes *E. faecium* dominance in the gut of mice was shown to diminish the mucus layer, alter intestinal architecture, change the mucosal microbiota, and deform E-cadherin adherens junctions. Despite these changes, direct attachment of *E. faecium* to IECs or its intestinal translocation was not observed (Hendrickx et al. 2015), unlike *E. faecalis* (Steck et al. 2011).

When the mucosal layer is compromised, enterococci may directly interact with the gut barrier by adhering to IECs (Fig. 3). *In vitro* studies have shown that glycosaminoglycans, such as heparin and heparan sulfate, are critical host receptors facilitating *E. faecalis* adhesion to colonic cells (Sava et al. 2009). Preincubation of colonic (Caco-2) cells with the enterococcal glycolipid diglucosyldiacylglycerol (DGDAG), a precursor of LTA, inhibited bacterial binding (Sava et al. 2009, Nuri et al. 2015). Consistent with this, mutants lacking genes essential for DGDAG synthesis showed decreased adherence to Caco-2 cells and reduced bacteremia in a mouse model (Theilacker et al. 2009, 2011). These results suggest that epithelial cells interact with DGDAG via heparin/heparan sulfate, aiding *E. faecalis* in attachment to colonic epithelia, a crucial initial step in gut translocation. Further research has shown that DGDAG synthesis is upregulated upon coculture with colonic cells *in vitro*. Additionally, the absence of DGDAG or other glycolipids significantly impairs *E. faecalis*' ability to translocate through intestinal monolayers in a two-chamber transcytosis model (Ramos et al. 2022). Other intrinsic factors also play a role in enterococcal translocation (Zeng et al. 2004, Maharshak et al. 2015, Shogan et al. 2015, Ramos et al. 2019, Fine et al. 2020). Among them, genes involved in EPA synthesis, like *epaX*, are required for efficient migration through intestinal epithelial barriers *in vitro* (Zeng et al. 2004, Ramos et al. 2019, Ramos et al. 2021). Immunofluorescence microscopy showed *E. faecalis* forming biofilm-like aggregates covered by exopolysaccharides, which localized with the epithelial actin cytoskeleton during translocation. These polymers were not detected when Δ*epaX* strains were used (Ramos et al. 2019). Thus, matrix-covered enterococcal aggregates might develop during attachment and migration across intestinal barriers *in vivo*. Further studies are needed to understand the role of EPA and other enterococcal polysaccharides in the translocation of these pathobionts in susceptible hosts.

Gut bacteria may disrupt tight or adherens junctions, enhancing barrier permeability through microbial factors and inflammatory responses (Fine et al. 2020). In a pancreatic sepsis model, *E. gallinarum* translocation to extraintestinal sites was linked to sepsis, partly dependent on mucosal TLR2 (Kamdar et al. 2013, Soares et al. 2017). *E. faecalis* OG1RF-derived GelE disrupted the epithelial barrier by degrading E-cadherin (Fig. 3 and Table 1) *in vitro*, contributing to intestinal inflammation in an IL-10-deficient mouse model of colitis (Steck et al. 2011). GelE secreted by *E. faecalis* V583 increased permeability in colonic epithelia of wild-type but not in PAR2 (protease-activated receptor 2)-deficient mice (Maharshak et al. 2015). It was proposed that enterococci may disrupt epithelial tight junctions via PAR2 activation, thereby exposing E-cadherin to GelE and contributing to barrier disruption (Maharshak et al. 2015). Moreover, high collagenase-producing *E. faecalis* strains have been associated with anastomotic leaks through GelE- and SprE-mediated depletion of intestinal collagen, followed by the activation of tissue MMP9, degrading the host extracellular matrix (Shogan et al. 2015). GelE has also been shown to degrade the gastrointestinal hormone glucagon-like peptide-1, GLP-1, which regulates gut glucose homeostasis (LeValley et al. 2020). Both findings link enterococcal overgrowth and tissue integrity disruption during GIT dysbiosis. Notably, in a ceftriaxone-induced mouse dysbiosis model, *E. faecalis* translocated to extraintestinal sites without causing intestinal pathology or altering tight junction protein expression or gut permeability (Chakraborty et al. 2018), suggesting that translocation may also occur via luminal interepithelial DCs and/or phagocytosis by lamina propria macrophages into the bloodstream or lymphatic system (Chakraborty et al. 2018). Indeed, recent work by Jennings et al. demonstrated that depletion of colonic phagocytes resulted in the reduction of *E. faecalis* OG1RF dissemination to the gut-draining mesenteric lymph nodes (Jennings et al. 2024). Additionally, goblet cell-associated passages or endocytosis by epithelial cells could serve as entry points into the lamina propria (Kalischuk et al. 2009, Wu et al. 2014, Knoop et al. 2015). Further research is crucial to fully understand the mechanisms of enterococcal egress from the gut.

Enterococcal egress from the GIT and immediate interactions with myeloid cells

The dissemination of gut-resident enterococci to distant sites such as peripheral blood, liver, kidney, brain, and heart is a complex process influenced by the ability of these bacteria to breach the lamina propria and access either the gut vascular barrier (GVB) or the gut lymphatic barrier (GLB). Both barriers are composed of endothelial cells connected by tight and adherens junctions (Spadoni et al. 2015, Kao and Kline 2019, Iida et al. 2021, Old-

berg and Rasmussen 2021). A breach in the GVB facilitates bacterial spreading to the liver (Fig. 3), whereas breaching the GLB can lead to dissemination to the mesenteric lymph nodes (MLNs) (Fine et al. 2020). Many enteric pathogens exhibit a preference for the lymphatic system as an escape route from the intestinal lumen and its underlying lamina propria (Magold and Swartz 2022). The gut-associated lymphatic system, particularly the M cells within the follicle-associated epithelium, allows for antigen exposure and directly shuttles translocating bacteria to the underlying Peyer's patches and/or gut-associated lymphoid tissues (GALTs). Once in the lymphatic system, bacteria can move to MLNs and potentially enter the bloodstream (Magold and Swartz 2022). *E. faecalis* can disseminate from the colon to colon-draining MLNs (Jennings et al. 2024). However, this dissemination route is not always linear, as some host cells may bypass the lymphatic vasculature to access the blood directly. For instance, although DCs can act as vehicles for enteric bacteria, navigating lymphatic vessels towards the MLNs, pathogens like *Salmonella* sp. secrete intracellular factors that redirect DCs toward the blood endothelium, facilitating systemic dispersal (Vazquez-Torres et al. 1999).

Robust innate immune responses typically eliminate low-level bacterial dissemination to the bloodstream. However, enterococci possess mechanisms to resist phagocytic killing, allowing for intracellular survival and further dissemination to and persistence in the bloodstream, leading to bacteremia (Smith and Nehring 2024). The following sections discuss the specific mechanisms by which enterococci interact with myeloid cells, detailing how these interactions enable bacteria to evade the immune system, survive intracellularly, and spread to distal organs.

Interaction with macrophages

Macrophages in the intestinal lamina closely interact with luminal bacteria, surveilling those that could breach the epithelial barrier. These phagocytes recognize conserved bacterial patterns such as LTA via PRRs, including surface TLRs and NOD-like receptors (NLRs) within the cytosol (Smith et al. 2011). Hence, upon detecting bacteria such as *E. faecalis* transversing the intestinal epithelium, macrophages engulf them and initiate an inflammatory response, ultimately leading to microbial clearance (Smith et al. 2011). Failure to do so can result in systemic infections and bacterial colonization of distant organs (Fig. 3).

Enterococci have developed resistance mechanisms against innate immune effectors like macrophages (Gentry-Weeks et al. 1999, Baldassarri et al. 2001, Baldassarri et al. 2005, Zou and Shankar 2016, Polak et al. 2021, Jennings et al. 2024, Norwood et al. 2024). The composition and dynamics of the enterococcal cell envelope play a crucial role in this resistance. For instance, several *E. faecalis* clinical isolates produce a capsular polysaccharide that masks opsonic C3 molecules from recognition by phagocytes (Thurlow et al. 2009). Additionally, enterococcal glycolipids may inhibit non-opsonic phagocytosis (Theilacker et al. 2011). The production of cell wall-anchored EPA by *E. faecalis* has also been linked to increased resistance to macrophage phagocytosis (Fig. 3; Teng et al. 2002, Prajsnar et al. 2013, Norwood et al. 2024). Smith et al. (2019) demonstrated that the absence of *epaX* significantly increased *E. faecalis* V583 uptake by macrophages compared with the wild-type strain. Supporting this study, recent findings showed that macrophage phagocytosis of an *E. faecalis* OG1RF mutant, lacking the cell-exposed EPA decorations, was restored to wild-type levels by complementing this strain with structurally distinct decorations from the V583 strain, and vice versa. (Norwood et al. 2024). EPA decorations thus seem to aid immune evasion through a conserved mechanism across different strains. Furthermore, the efficiency of *E. faecalis* uptake by macrophages is further decreased by the autolysin AtlA, which inhibits the formation of long enterococcal chains that are more easily phagocytosed (Salamaga et al. 2017). Conversely, enterococcal cells lacking EPA decorations form aggregates that demonstrate enhanced phagocytosis in vitro through a mechanism independent of lipoprotein recognition by macrophages (Norwood et al. 2024). Further investigation is needed to understand the roles of opsonic and non-opsonic phagocytosis and to evaluate whether enhancing these pathways can improve enterococcal clearance.

The uptake of enterococci by phagocytes does not always lead to intracellular bacterial clearance, as they can survive inside macrophages (Gentry-Weeks et al. 1999, Zou and Shankar 2016, Polak et al. 2021). Indeed, it was shown that glucose-grown enterococcal isolates, which form aggregates (biofilms) containing extracellular polysaccharides, can survive inside rat macrophages for up to 48 h. In contrast, polysaccharide-biofilm-deficient strains are killed within 24 h post-infection (Baldassarri et al. 2001, 2004, 2005). During the infection process, electron microscopy revealed that enterococci adhered to macrophages and entered through small ruffles encircling the bacterial cells (Baldassarri et al. 2005). *E. faecalis* was found as single or multiple cells within intact phagocytic vacuoles, where the production of polysaccharide-containing biofilms aids in their survival for up to 24 h (Fig. 3; Gentry-Weeks et al. 1999, Baldassarri et al. 2005). Based on these findings, it was proposed that entry of the biofilm/polysaccharide-positive strain is mediated by receptor-mediated endocytosis, dependent on microtubule reorganization, microfilament polymerization, and activation of protein kinases such as PI3K, as inhibition of the latter reduces bacterial binding (Baldassarri et al. 2005). Notably, macrophages engulfing *E. faecalis* upregulate anti-apoptotic and pro-survival pathways by increasing phosphorylation of PI3K and decreasing cleaved caspase-3 activity, thus enhancing bacterial persistence (Zou and Shankar 2014). Additionally, *E. faecalis*' persistence within macrophages is partly due to its resistance to low pH levels and its ability to delay phagosome acidification, the primary mechanism macrophages use to eliminate ingested microbes (Zou and Shankar 2016). Together, these findings suggest that early post-infection, *E. faecalis* uses multiple mechanisms to persist and multiply within macrophages, blocking clearance via regulated cell death.

The ability of macrophages to acquire phenotypic plasticity and shift polarization states is crucial during infections and tissue repair (Das et al. 2015). Recent studies showed that *E. faecalis* can polarize macrophage precursors to express reduced pro-inflammatory cytokines such as IL-1β and IL-12 while increasing their capacity to produce immunoregulatory cytokines such as IL-10 (Mohamed Elashiry et al. 2021). Reduced IL-1β production in this setting may compromise neutrophil recruitment and the initiation of adaptive immunity (Sahoo et al. 2011, Ratner et al. 2016). Additionally, enterococcal membrane vesicles containing DNA have been shown to induce type I interferon (IFN) production in bone marrow-derived macrophages by activating the cGAS-STING pathway (Erttmann et al. 2022), suggesting that intestinal phagocytes could undergo similar responses in the lamina propria (Fig. 3).

Unlike conventional macrophages, intestinal macrophages are generally anti-inflammatory (Yip et al. 2021), producing cytokines such as IL-10 to mitigate gut inflammation and promote the expansion of regulatory T cells (Tregs; Yip et al. 2021). This raises the question of whether these tolerogenic macrophages might be less effective at eliminating translocating bacteria, such as

E. faecalis, potentially allowing these bacteria to be carried as cargo and facilitating their translocation to secondary lymphoid tissues, such as the mesenteric lymph nodes. Indeed, using a ceftriaxone-induced dysbiosis mouse model, Jennings and colleagues showed that *E. faecalis* OG1RF utilizes monocyte-derived CX3CR1-expressing phagocytes to move to the MLNs (Fig. 3). Notably, rectal administration of clodronate liposomes, which depletes colonic phagocytes, prevented *E. faecalis* dissemination to the MLNs, independent of CCR7 expression—a key receptor DC migration to lymphoid organs. These findings suggest that *E. faecalis* transport to the MLNs is facilitated by CX3CR1-expressing macrophages rather than by CCR7-mediated DC migration.

E. faecalis dissemination to the MLNs requires prolonged intracellular survival, regulated by oxidative stress genes such as manganese-containing superoxide dismutase (*sodA*). *E. faecalis* mutants lacking SodA exhibit reduced survival within macrophages and consequently lessened dissemination to the MLNs. This indicates that SodA-mediated intracellular survival is crucial for *E. faecalis* dissemination via monocyte-derived CX3CR1-expressing phagocytes (Jennings et al. 2024). While the mechanism by which *E. faecalis* disseminates to the MLN is partly understood, the process by which it translocates to distal organs such as the liver and spleen remains to be elucidated. Further research is necessary to understand how *E. faecalis* disseminates from the MLNs and to determine whether targeting bacterial factors such as SodA could improve enterococcal clearance and dissemination.

Interaction with neutrophils

During primary infection, neutrophil attraction to the site of injury aids in rapid clearance of enterococci (Leendertse et al. 2009). Opsonized bacteria engage the neutrophils' complement receptor (CR3), activating phagocytosis and phagosome acidification, leading to bacterial elimination through the complement-mediated pathway (van Kessel et al. 2014). Neutrophils primarily rely on phagocytosis and reactive oxygen species production to eliminate *E. faecalis* (Kao et al. 2023). However, *E. faecalis* does not induce neutrophil extracellular trap formation (NETosis), a mechanism typically used by these myeloid cells to kill extracellular bacteria (Kao et al. 2023), suggesting that *E. faecalis* attenuates PMN-mediated responses.

The AS-Asc10- from *E. faecalis* mediates effective bacterial adhesion to human neutrophils by enhancing opsonin-independent bacterial binding to this myeloid subset (Vanek et al. 1999). This process depends on the CR3 receptor on the surface of human neutrophils, as adhesion of *E. faecalis* was inhibited by 85% when a CR3-blocking antibody was used or with neutrophils from patients with leukocyte adhesion deficiency (Vanek et al. 1999). While both complement- and AS-mediated phagocytosis result in *E. faecalis* internalization by neutrophils, further studies have shown that AS-internalized *E. faecalis* exhibits resistance to phagocytic killing, with intact plasma membranes inside the neutrophil phagosome (Rakita et al. 1999). Phagosome acidification is essential for killing intracellular pathogens, with pH normally dropping as low as 4.5 (Rakita et al. 1999). Notably, the phagosomal pH of neutrophils containing AS-bearing *E. faecalis* was less acidic than that of neutrophils containing opsonized *E. faecalis* (Rakita et al. 1999). This suggests that AS-bearing *E. faecalis* can evade neutrophil killing by compromising phagosome acidification, reducing bacterial killing, and enhancing bacterial survival within host tissues.

Venturing beyond the GIT

Enterococci in the heart

The endothelium, located in the inner layer of the heart chambers, valves, and blood vessels, becomes a target for circulating enterococci potentially originating from sites like the GIT, urogenital tract, or oral cavity (Fig. 4; Silva et al. 2017, Liesenborghs et al. 2020, Del Giudice et al. 2021). These bacteria can establish on susceptible endothelial surfaces, particularly on the endocardium of previously disturbed cardiac valves, contributing to IE (Holland et al. 2016, Liesenborghs et al. 2020). Enterococci are implicated in up to 20% of IE cases, with *E. faecalis* identified as the primary causative agent (Chirouze et al. 2013, Amat-Santos et al. 2015, Holland et al. 2016, Bussani et al. 2019, Dahl et al. 2019).

The conventional IE model involves distinct stepwise events after endothelial surface perturbations: (i) recruitment of clotting factors (fibrin) and platelets, forming sterile NBTE vegetations; (ii) bacterial adhesion leading to clot colonization; and (iii) development of bacterial microcolonies/biofilms on and within the nascent septic vegetation (Fig. 4; Holland et al. 2016, Liesenborghs et al. 2020, Barnes et al. 2021). Most IE animal models consist of introducing a catheter through the aortic valve to mechanically disrupt the endothelial surface, exposing the matrix components of the subendothelial layer (fibrinogen, collagen, laminin, or fibronectin) and subsequent formation of NBTEs, mimicking the infection process observed in patients with endocarditis (Holland et al. 2016, Goh et al. 2017, Liesenborghs et al. 2020, Barnes et al. 2021). Although bloodstream enterococci could bind to a pre-existing NBTE prior to forming an infected vegetation (McGowan and Gillett 1980, Liesenborghs et al. 2020), recent research revealed that *E. faecalis* can also establish cardiac surface colonization in the absence of pre-existing damage or NBTE formation in a rabbit endovascular infection model (Barnes et al. 2021). SEM analyses showed *E. faecalis* microcolonies attached through the cardiac endothelium that were indistinguishable from those established in the hearts of rabbits that received mechanical interventions (Barnes et al. 2021). Approximately 50% of endocarditis patients with structurally normal heart valves show no inherent vulnerability to cardiac tissue perturbation, suggesting that endothelium inflammation, irrespective of damage, sensitizes this layer to infection (Que and Moreillon 2011, Werdan et al. 2014, Olmos et al. 2017). Whether NBTE formation is imperative for enterococcal IE development remains debated, as direct bacterial attachment to the endothelium could initiate/serve as a reservoir for subsequent infection, akin to other bacterial species (Hamill et al. 1986, Yao et al. 1995).

Enterococcal adherence to the endocardium

Efficient bacterial adhesion to damaged or inflamed cardiac surfaces, achieved through multiple mechanisms (Fig. 4), is pivotal in IE to overcome shear stress from the high blood flow passing through the valves (Midha et al. 2017). *E. faecalis* binds directly to the endothelium or NBTE matrix components (Scheld et al. 1985, Munita et al. 2012, Barnes et al. 2021), likely through adhesion proteins (Fig. 4). A well-characterized family of adhesins is AS (Table 1), of which three have been the most studied—Asc-10, Asa1, and Asp1—encoded on plasmids pAD1, pCF10, and pPD1, and sharing >90% identity (Chuang et al. 2009). Asc10 expression was observed in a pCF10-carrying strain when cultured in blood *in vitro* and during IE in a rabbit model *in vivo* (Hirt et al. 2002). While not deemed essential for inducing IE (Schlievert et al. 1997), AS synthesis may be implicated in the tissue binding process, as the expression of pCF10 increases enterococcal cell hydrophobic-

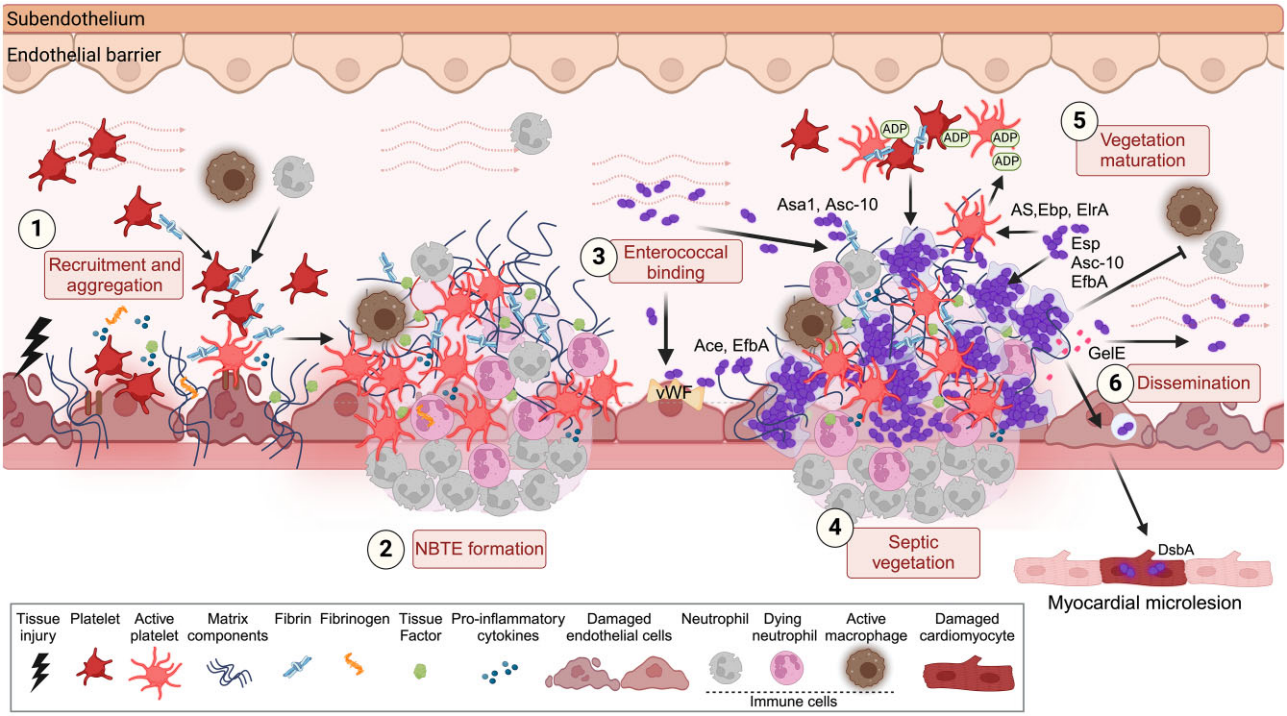

Figure 4. Interactions between host cells and enterococci during IE. The endothelium, located in the inner layer of the heart chambers, valves, and blood vessels, becomes susceptible to IE when injured or perturbed, exposing subendothelial matrix components like fibrinogen, collagen, laminin, and fibronectin. (**1**) Platelets aggregate at the damaged endothelium, inducing cytokine production and upregulating tissue factor and fibrinogen. Active platelets produce fibrin, which stimulates further aggregation and serves as a scaffold for additional platelets and immune cells, promoting inflammation and forming a sterile NBTE vegetation (**2**). Bloodstream enterococci may bind to pre-existing NBTE or directly to damaged or inflamed cardiac surfaces, overcoming shear stress from high blood flow via different mechanisms (**3**): The von Willebrand Factor (vWF) mediates bacterial binding to the endocardium, acting as a bridge between bacteria and host cells; Ace and EfbA (fibronectin-binding protein) promote binding to subendothelial components such as collagen, laminin, and fibronectin; and enterococcal Asc-10 and Asa1 enhance attachment to fibrin by increasing cell hydrophobicity, all leading to enterococcal colonization and biofilm formation within the nascent septic vegetation (**4**). The maturation of the infected vegetation involves cycles of fibrin-platelet deposition, with bacteria stimulating platelet aggregation. *Enterococcus faecalis* interacts with platelets through envelope components such as AS, Ebp pili, and ElrA, promoting further aggregation and the extracellular release of adenosine diphosphate (ADP) from dense platelet δ-granules. Esp promotes enterococcal cell-cell aggregation by binding to cell envelope components like lipoteichoic acid (LTA) and, together with Asc-10 and EfbA, influences enterococcal biofilm maturation and growth. This septic vegetation growth and biofilm formation protect enterococci from antimicrobials to immune cells (**5**). GelE-mediated degradation of fibrin-rich matrices facilitates bacterial spread from vegetations to adjacent or distal sites (**6**). *Enterococcus faecalis* may invade endothelial cells via receptor (clathrin)-mediated endocytosis, further contributing to disease progression. Myocardial microlesions can result from the spread of infection, with the disulfide bond-forming protein A (DsbA) being necessary, enhancing cell death and suppressing the immune response.

ity and enhances attachment to fibrin *in vitro* (Hirt et al. 2000). Additionally, Asc-10 has been shown to promote the formation of larger infective vegetations and higher bacterial loads, exacerbating the severity of experimental IE (Hirt et al. 2002, Chuang et al. 2009). This may result from these adhesins accelerating cell-cell aggregation by binding to cell envelope components like LTA. Of note, strains with altered LTA demonstrated significantly reduced virulence in the IE rabbit model, and the absence of AS and LTA rendered the strains completely avirulent (Schlievert et al. 1998). LTA binds to the membrane by a glycolipid anchor (Reichmann and Grundling 2011), which is synthesized by the glycosyltransferases BgsA and BgsB in *E. faecalis* (Theilacker et al. 2011). In fact, *E. faecalis* 12030 lacking *bgsA* or *bgsB* exhibited reduced endocardial lesions and bacterial loads in the vegetation (Haller et al. 2014), highlighting the central role of cell envelope components like LTA in IE.

Adhesins of the MSCRAMM family, such as Ace in *E. faecalis* (Nallapareddy et al. 2000) and Acm in *E. faecium* (Nallapareddy et al. 2008), are considered crucial for initial attachment to cardiac tissue. Ace demonstrated high binding affinity to collagen (types I and IV) and laminin (Nallapareddy et al. 2000, Singh et al. 2010), and in rat models of enterococcal IE, mutants lacking Ace or Acm showed significant attenuation compared to wild-type strains during early infection stages (Nallapareddy et al. 2000, 2008, Singh et al. 2010). Other noteworthy extracellular matrix-binding proteins include *E. faecalis* EfbA (Enterococcal Fibronectin-Binding Protein) and its homolog in *E. faecium* (Fnm), which have been demonstrated to interact with immobilized fibronectin, collagen, or laminin in a concentration-dependent manner *in vitro* (Torelli et al. 2012, Singh et al. 2015, Somarajan et al. 2015). Consequently, enterococci devoid of either of these proteins reveal attenuation in IE (Singh et al. 2015, Somarajan et al. 2015), and both passive and active immunization against Ace or EfbA reveal robust protection and significantly reduce infection rates (Singh et al. 2010, 2015). In addition to these proteins, the fibrinogen adhesins Fss1, Fss2, and Fss3 have been shown to mediate adherence of the blood isolate *E. faecalis* V583 (Sahm et al. 1989) to host extracellular matrix proteins (Sillanpää et al. 2009), highlighting a repertoire of potential binding mechanisms for initial interactions with the cardiac environment *in vivo* (Fig. 4).

Understanding how enterococci attach to tissues without exposed sub-endothelium or NBTE components is crucial. The von Willebrand factor (vWF) mediates bacterial binding to the endocardium (Pappelbaum et al. 2013, Claes et al. 2014). During inflam-

mation, vWF transitions from circulating in the bloodstream to an endothelium-bound form, where it unfolds under shear stress to reveal the vWF A1 domain, allowing bacterial binding (Huck et al. 2014, Liesenborghs et al. 2020). Thus, vWF acts as a bridge between bacteria and host cells (Fig. 4), enabling resistance to blood flow (Claes et al. 2014). *E. faecalis* may utilize leucine-rich protein A (ElrA) for direct interaction with the vWF domain (Jamet et al. 2017). vWF can bind to platelets through the GP1b receptor, slowing platelet movement and promoting interactions with molecules in the endothelium or subendothelial layer (Liesenborghs et al. 2020). *E. faecalis* has been shown to adhere to human platelets *in vitro* via the endocarditis- and biofilm-associated pilus (Ebp; Table 1), which may also facilitate bacterial binding to vegetation on heart valves. Mutants lacking Ebp exhibit reduced virulence in an experimental endocarditis model (Nallapareddy et al. 2006, 2011a). Consequently, immunization against pilus components like EbpC reduces susceptibility to IE (Pinkston et al. 2014). Ebp was found to be highly expressed on the surface of rat endocarditis vegetations (Nallapareddy et al. 2011a); however, Pili expression was limited to a subset of cells *in vitro*, suggesting a nuanced role at different infection stages (Nallapareddy et al. 2011a, Manias and Dunny 2018). Given that Ebp and Ace were found genetically conserved among *E. faecalis* isolates from various origins (Nallapareddy et al. 2011a), further research is needed to unravel the regulatory mechanisms of these factors *in vivo* to clarify their roles in the development of enterococcal IE.

Enterococcal-driven maturation of the infective vegetation

A key stage in IE is the maturation of the infected vegetation, involving cycles of fibrin-platelet deposition, with bacteria stimulating platelet aggregation (Fig. 4; Holland et al. 2016, Brai et al. 2023). Enterococci-induced platelet aggregation has been shown to require the extracellular release of adenosine diphosphate from dense platelet δ-granules *in vitro* (Usui et al. 1991). Additionally, *E. faecalis* mediates platelet aggregation through cell envelope components such as AS, Ebp pili, and the ElrA protein (McCormick et al. 2002, Nallapareddy et al. 2011a, Jamet et al. 2017). Prophage-associated genes have also been implicated in the development of infectious vegetation (Laumay et al. 2019). In particular, prophages pp1, pp4, and pp6 in *E. faecalis* V583, homologous to the prophage φSM1 platelet-binding proteins PblA and PblB in *Streptococcus mitis*, have been shown to be essential for adhesion to human platelets (Bensing et al. 2001, Matos et al. 2013). PblA and PblB proteins interact with α2–8-linked sialic acids on ganglioside GD3 for platelet adhesion; loss of these proteins reduces platelet binding *in vitro* (Mitchell and Sullam 2009). Notably, it has been shown that enterococcal genetic diversity is high within the same heart valve during prolonged IE and that the capacity for platelet aggregation varies among enterococcal isolates, with some strains demonstrating an inability to promote the aggregative phenotype (Johansson and Rasmussen 2013, Royer et al. 2021). Supporting this, Hannachi et al. (2020) revealed that *E. faecalis*-formed infective endocardial vegetations are composed of abundant erythrocytes rather than high proportions of platelets and fibrin networks, as seen in other gram-positive pathogens.

Platelets respond to injured endothelium by generating cytokines and upregulating tissue factors, initiating an inflammatory response (Fig. 4). Active platelets produce procoagulant molecules that further stimulate their aggregation. Fibrin then serves as a scaffold for other incoming platelets and leukocytes, promoting inflammation (Flick et al. 2004). In IE, this hemostasis is enhanced by bacterial infection (Panizzi et al. 2011), leading to vegetation growth and biofilm formation (reviewed in Lerche et al. 2021), which protect bacteria and make them more resistant to antimicrobials and immune cells (Moreillon et al. 2002, Liesenborghs et al. 2020, Barnes et al. 2021). Platelets can also enhance biofilms during IE (Jung et al. 2012). *E. faecalis* OG1RF can form microcolonies on the injured and intact endocardium of infected white rabbits that exhibited architectures similar to biofilms found in other *in vivo* and *in vitro* models (McCormick et al. 2002, Barnes et al. 2017, Ramos et al. 2019). Few enterococcal factors involved in biofilm formation during IE have been identified. The ArhC transcription factor is necessary for early attachment and biofilm biomass accumulation *in vitro*, and its absence in *E. faecalis* attenuates endocarditis (Frank et al. 2013). Asc10 accelerates the development of larger microcolonies with abundant exopolymeric matrices via cellular aggregation (Chuang-Smith et al. 2010). A non-piliated EbpA-deficient strain produces biofilms with a density similar to a non-biofilm producer (Nallapareddy et al. 2006), and an EfbA mutant forms biofilms with lower density compared to its parental strain (Singh et al. 2015).

Colomer-Winter et al. (2018) proposed that *E. faecalis* proliferation in valves is sustained by the continuous nutrient supply from the bloodstream. They observed that the stringent response, orchestrated by (p)ppGpp and typically triggered by nutrient deprivation, remained inactive in heart valve-associated *E. faecalis*. Deleting the gene encoding the bifunctional (p)ppGpp synthase/hydrolase Rel significantly hindered endocardial colonization in a mouse model. While (p)ppGpp was non-essential for inducing enterococcal IE, regulating (p)ppGpp levels was crucial for valve colonization, indicating that the pathophysiological state influences adaptation and colonization (Colomer-Winter et al. 2018). Similarly, *E. faecium*'s physiological state affects its ability to cause IE. The absence of CcpA, a global carbon metabolism regulator, impacted *E. faecium* growth in an IE rat model, and its colonization on aortic valves was outcompeted during coinfection with its parental strain (Somarajan et al. 2014). Likewise, lacking *bepA*, which encodes a carbohydrate phosphotransferase system permease, rendered *E. faecium* unable to outcompete its wild-type strain in a mixed IE model (Paganelli et al. 2016). Nonetheless, the specific impact of basal levels of (p)ppGpp or carbon metabolism on the pathogenicity of enterococci in infective endocarditis requires further investigation.

Invasion of cardiac tissue

Local tissue invasion and abscess formation are characteristic features of IE caused by various gram-positive bacteria (Moreillon et al. 2002). *E. faecalis* adheres to and resides within the vacuoles of human umbilical vein endothelial cells (HUVEC) cells, surviving within endothelial cells via receptor (clathrin)-mediated endocytosis in a cytochalasin-D and colchicine-dependent manner (Fig. 4; Millan et al. 2013). However, the specific bacterial or endothelial components facilitating this internalization remain unknown. Kline and coworkers demonstrated that *E. faecalis* is internalized into keratinocytes through single membrane-bound compartments, where it can persist and manipulate the endosomal pathway (da Silva et al. 2022). Further research should determine whether enterococcal endothelial internalization occurs via micropinocytosis, as in keratinocytes (da Silva et al. 2022).

The ability of certain bacteria to invade and persist within the endothelium significantly contributes to the progression of IE, resulting in endovascular infections characterized by endothelial destruction, tissue invasion, and dissemination (Moreillon et al.

2002, Holland et al. 2016). *E. faecalis* production of GelE, rather than SprE, is suggested to facilitate bacterial dissemination from vegetations through GelE-mediated degradation of fibrin-rich matrices (Fig. 4; Waters et al. 2003, Thurlow et al. 2010). Thurlow et al. (2010) demonstrated that vegetation on valves infected with *E. faecalis* V583 lacking GelE had a 10-fold thicker fibrin-containing matrix and increased bacterial burden compared to those colonized by the parent strain. GelE presence is expected to enable enterococcal dissemination by slightly thinning valve biofilms, allowing the walled-off vegetation to embolize and spread to adjacent or distal sites, causing abscesses and tissue death.

Internalization of *E. faecalis* by endothelial cells has been shown to induce apoptosis at high bacterial loads *in vitro* (Millan et al. 2013). Bacterial endothelial internalization can result in complex infections, which can culminate in the development of myocardial abscesses by direct extension (Trifunovic et al. 2018). Myocardial abscesses in IE result from the spread of infection from the valve to perivalvular structures, forming perivalvular abscesses (Brown and Garsin 2020). In contrast, *E. faecalis* typically does not form myocardial abscesses but promotes microlesions in the myocardium, often associated with a suppressed immune response (Brown and Garsin 2020). Brown et al. (2021) observed that *E. faecalis* OG1RF causes cardiac microlesions during severe bacteremia in mice, with the disulfide bond-forming protein A (DsbA) being necessary (Brown et al. 2021). DsbA was shown to enhance cell death and suppress the immune response during *E. faecalis* infection of a cardiomyocyte cell line (Brown et al. 2021), highlighting the role of bacterial immune evasion in cardiac microlesion formation (Fig. 4).

Despite extensive research on the interactions between enterococci and cardiac tissues, relatively few studies have examined the effects of enterococci after they translocate to other host sites, such as the kidney or liver, and their subsequent impact on host health. In the following section, we will summarize key findings related to these interactions that have been described so far.

Enterococci in the liver

Translocated bacteria, or PAMPs, from the GIT can enter the portal circulation and reach the liver, triggering an innate immune response. This hepatic inflammation contributes to liver injury and disease (see review Chopyk and Grakoui 2020). Factors such as fecal dysbiosis, small intestinal bacterial overgrowth, gut epithelial barrier dysfunction, and increased permeability are crucial in promoting chronic liver disease (Chopyk and Grakoui 2020). For instance, chronic alcohol abuse disrupts tight junction integrity, increasing intestinal permeability and creating a dysbiotic microbiota (Imhann et al. 2016, Llorente et al. 2017, Duan et al. 2019). Consequently, this is linked to liver disorders ranging from steatosis to hepatitis, cirrhosis, and cancer (Chopyk and Grakoui 2020). Notably, studies in experimental ALD (alcoholic liver disease) mice and patients show that the translocation of intestinal *E. faecalis* to the liver exacerbates ethanol-induced liver inflammation and hepatocyte damage (Llorente et al. 2017, Duan et al. 2019). Moreover, conditions that suppress gastric acid, like long-term use of proton pump inhibitors, enhance enterococcal gut expansion and translocation to the liver. Hence, promoting the progression of ALD, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis progression in both mouse models and humans (Llorente et al. 2017). These studies indicate that viable enterococci reach the liver and engage TLRs on Kuffer cells (liver-resident macrophages), leading to IL-1β secretion, inflammation, and hepatic tissue damage (Llorente et al. 2017).

Iwasa and collaborators have identified factors related to *E. faecalis* in chronic liver diseases, including ALD (Iwasa et al. 2022). They found elevated levels of antibodies specific to the enterococcal capsule in the serum of patients with advanced chronic liver disease. These anti-capsule antibodies may reflect the status of the liver-gut axis. Notably, treatment with rifaximin to reduce bacterial loads decreased both symptoms and antibody titers (<0.018) in patients, leading to increased survival rates. This suggests that capsule expression plays a crucial role in the progression of liver disease (Iwasa et al. 2022).

The severity and mortality of alcoholic hepatitis have been linked to the presence of *E. faecalis* expressing the exotoxin cytolysin (Table 1 and Fig. 3; Duan et al. 2019). Approximately 80% of alcoholic hepatitis patients had increased endogenous *E. faecalis* in their feces, with 30% having cytolysin-positive enterococci, which were absent in healthy individuals' fecal samples. Notably, >80% of cytolysin-positive patients with alcoholic hepatitis died within 180 days, indicating that *E. faecalis* can worsen liver disease outcomes (Duan et al. 2019). Moreover, whole-genome sequencing of 93 isolates from hepatitis patients showed broad phylogenetic diversity of cytolysin-positive *E. faecalis* in those with alcoholic hepatitis, with no correlation found between disease severity and other antimicrobial resistance or virulence genes (Duan et al. 2019). The same study showed that mice gavaged with cytolysin-positive *E. faecalis* [strain FA2–2(pAM714)] and fed ethanol developed more severe liver injury and produced more proinflammatory factors, such as IL-1β, compared to controls (Duan et al. 2019). Of note, the exacerbation caused by cytolytic endogenous *E. faecalis* seems specific to ALD, as another study found no relationship between the presence of the exotoxin in patient feces and non-alcoholic fatty liver disease outcome (Lang et al. 2020).

Pure bioactive cytolysin peptides were shown to cause a dose-dependent increase in cell death in hepatocytes from ethanol-fed mice compared to controls, likely mediated by pore formation resulting in cell lysis (Duan et al. 2019). Parenchymal hepatocytes, comprising 70%–85% of the liver, are crucial for nutrient metabolism and protein synthesis. Additionally, hepatocytes contribute to innate immunity by producing antimicrobial proteins that kill or opsonize bacteria, assist with phagocytosis, or sequester iron essential for bacterial growth (Zhou et al. 2016). *E. faecalis* can internalize, survive, replicate, and form small aggregates in liver hepatocytes both *in vitro* and *in vivo* (Nunez et al. 2022). Inhibition of innate liver immunity reduces macrophage and neutrophil populations, coinciding with the formation and spread of enterococcal aggregates (Nunez et al. 2022). The exact mechanisms of *E. faecalis* invasion and its effects on liver homeostasis remain unclear. Further research is needed to understand how enterococci adhere to and invade hepatocytes. Enterococcal intracellular survival and replication are widespread, as seen in kidney cells and keratocytes (da Silva et al. 2022, Nunez et al. 2022). Future studies on the intracellular lifestyle of *E. faecalis* are warranted, considering the variety of organs it targets.

Translocation of intestinal microbiota has also been linked to hepatocellular carcinoma (HCC) development in a TLR4-dependent manner (Dapito et al. 2012). HCC is one of the leading causes of cancer-related death worldwide, and hepatitis C is considered a major risk factor. A recent study revealed an increased abundance of *gelE*-positive *E. faecalis* in patients with hepatitis C virus-related chronic liver diseases. Moreover, transplanting gut microbiota from *E. faecalis*-positive patients increased liver tumors in mice (Iida et al. 2021). The abundance of *gelE*-positive *E. faecalis* was associated with increased gut permeability and the number of liver tumors formed (Iida et al. 2021). However, the mechanisms

of *E. faecalis* translocation to the liver and interaction with liver immune cells remain largely unexplored.

Enterococci in the kidney

While uncommon, acute pyelonephritis (kidney infection) can occur hematogenously without an initial bladder infection, a condition known as a "descending" infection (Measley and Levison 1991, Kotanko et al. 2006). This occurs when blood-borne bacteria migrate into the kidneys, typically in immunocompromised patients or those with urethral obstructions (Bianchi-Jassir et al. 2017). Indeed, enterococci have been recovered from kidneys after systemic infections originating from gut translocation or intravenous inoculation, demonstrating a hematogenous route (Montgomerie et al. 1977, Archambaud et al. 2019).

Most research on enterococcal kidney infections uses animal models of ascending urinary tract infections (UTIs; Goh et al. 2017), which have clarified the complex interactions between enterococci and the kidney. Kau et al. (2005) demonstrated that *E. faecalis* can persist in the kidneys of infected mice for at least 2 weeks, eliciting an inflammatory response independent of TLR2 signaling. Furthermore, the absence of *E. faecalis* factors such as adhesins (Ace or Esp) or cell surface components (Epa or Ebp pili) results in reduced kidney infections in mouse models (Shankar et al. 1999, Singh et al. 2007, Lebreton et al. 2009, Nallapareddy et al. 2011b, Garsin et al. 2014). Enterococcal mutant strains lacking glycerol metabolism genes showed reduced kidney and liver colonization in mice seven days post-infection, suggesting that glycerol metabolism is necessary for bacterial persistence in these organs (Muller et al. 2015, Goh et al. 2017). However, studies investigating descending versus ascending routes of enterococcal kidney infection are lacking.

Pyelonephritis often develops from an ascending UTI (for comprehensive reviews, see references Flores-Mireles et al. 2015, Goh et al. 2017, Klein and Hultgren 2020). *E. faecalis* and *E. faecium* are common etiological agents of UTIs, particularly in chronically hospitalized patients, where factors such as obstruction, urinary catheterization, or instrumentation are prevalent (Ipe et al. 2013, Cai and Bartoletti 2017, Whiteside et al. 2018, Krawczyk et al. 2021). Enterococci can migrate from the GIT, perineum, or vagina to the urethra (urethritis), bladder (cystitis), and eventually the kidney (Flores-Mireles et al. 2015, Klein and Hultgren 2020). Once in the bladder, bacteria can attach to the urogenital tissues and form biofilms, resisting removal by the urine flow. If the inflammatory response fails to clear the infection, bacteria proliferate and produce toxins and enzymes that aid their survival (Mancuso et al. 2023). Hence, secreted gelatinases can act as immunomodulatory factors by cleaving complement components (C3, C3a, and C5a), helping bacteria evade the innate immune system (Codelia-Anjum et al. 2023).

Numerous virulence factors associated with UTIs caused by *E. faecalis* have been identified. Mutants lacking adhesion-promoting factors such as Esp, Ebp Pili, Ace, EfbA, and SrtC, envelope components like Epa, and elements such as MsrA, MsrB, SigV, and GrvR/EtaR show reduced virulence in the urinary tract (Goh et al. 2017). Additionally, a *dltA* gene mutant, crucial for D-alanylation of LTA in *E. faecalis* 12030, demonstrated enhanced colonization and adherence to human bladder carcinoma cells *in vitro* compared to the wild-type strain. Pre-treatment with purified LTA inhibited *dltA* mutant attachment to bladder cells in a dose-dependent manner, suggesting D-alanylation modulates the initial interaction with bladder tissues (Wobser et al. 2014). However, the precise role of D-alanylated LTA in the bladder or with urothelial cells remains unclear. While *E. faecium* is frequently isolated from hospital-acquired UTIs, it has been studied less extensively than *E. faecalis*. *E. faecium* uses surface proteins such as Esp and EmpABC pili to mediate colonization of the mouse urinary tract and shows a similar affinity for kidney colonization (Montgomerie et al. 1977, Leendertse et al. 2009, Sillanpää et al. 2010).

E. faecalis can establish in the urinary tract after catheterization, from which it can also ascend to the kidney in complicated infections (Flores-Mireles et al. 2015). Catheterization induces mechanical stress, leading to histological and immunological changes in the bladder, including inflammation, exfoliation, edema, and mucosal lesions in the uroepithelium and kidneys (Hooton et al. 2010, Codelia-Anjum et al. 2023). Hence, fibrinogen released during inflammation accumulates on the catheter, facilitating enterococcal adhesion, colonization, and biofilm formation and the development of catheter-associated UTIs (CAUTIs; Hooton et al. 2010, Flores-Mireles et al. 2015, Xu et al. 2017, Fiore et al. 2019, Codelia-Anjum et al. 2023). *In vitro* studies show that *E. faecalis* can attach to fibrinogen-coated catheters and thrive in urine with fibrinogen (Goh et al. 2017, Alhajjar et al. 2020). The absence of GelE and SprE results in attenuated CAUTI and defective biofilm formation. SprE, activated by a host trypsin-like protease, and treatment with inhibitors for both bacterial enzymes and host proteases, reduced catheter-induced inflammation and prevented the spread from the bladder to the kidney in a murine model (Xu et al. 2017). This indicates that GelE, SprE, and host proteases interact with fibrinogen, contributing to CAUTIs. For further information about CAUTIs, refer to this review (Flores-Mireles et al. 2015).

Enterococci in the vaginal tract

Originating from the GIT, *E. faecalis* can colonize the vaginal tract of healthy women (Ravel et al. 2011, Leyva-Gómez et al. 2019, Alhajjar et al. 2020). The prevalence of enterococci increases with a decline in the *Lactobacillus* population, leading to aerobic vaginitis, i.e. characterized by an inflammatory response (Donders et al. 2017, Kaambo et al. 2018). However, the molecular mechanisms enabling *E. faecalis* colonization and persistence in the vaginal tract remain elusive. Recent research using *in vitro* and *in vivo* models has identified factors contributing to *E. faecalis* vaginal adherence and persistence (Alhajjar et al. 2020). This study demonstrated that both strains V583 and OG1RF persist for ~11 days in a mouse model. Mutations in the Ebp pili reduced attachment to human vaginal and cervical cells *in vitro* but did not affect enterococcal establishment *in vivo*, suggesting that multiple factors are required for vaginal colonization (Alhajjar et al. 2020). Ethanolamine metabolism also provides a fitness advantage for enterococcal persistence in the vaginal tract, as a mutant in ethanolamine catabolism showed significantly reduced ability to colonize the vaginal epithelium (Alhajjar et al. 2020). Although ethanolamine might originate from other microbes or the vaginal epithelium, its roles in promoting virulence, commensalism, or modulating immune responses in the vaginal tract remain unclear.

Additionally, insertional mutations in type VII secretion system (T7SS) genes diminished late vaginal colonization by *E. faecalis* OG1RF and were necessary for better access to reproductive tract tissues (Alhajjar et al. 2020). This indicates that T7SS is involved in vaginal persistence and ascension in the female reproductive tract. Notably, bacterial T7SS mediates interbacterial competition (Spencer and Doran 2022). Upon phage induction, *E. faecalis* T7SS promoted the killing of *E. faecium*, *S. aureus*, and *Listeria monocytogenes* (Chatterjee et al. 2021), suggesting a role of this

secretion system in enterococcal competition and dominance in environments such as the vaginal tract or the intestine to promote dysbiosis. T7SS is also associated with bacterial-host interactions. In experimental meningitis models, T7SS increased neutrophil chemoattractant secretion and promoted brain endothelial cell death (Spencer et al. 2021). In *S. aureus*, T7SS modulated cytokine responses and reduced macrophage recruitment during murine blood infection (Anderson et al. 2017). Therefore, further investigation into the role of enterococcal T7SS in modulating host inflammatory responses and cell death pathways is warranted.

Persistent vaginal colonization with uropathogens often precedes the development of recurrent UTIs in women (Brannon et al. 2020). Currently, three transitional reservoirs are proposed to facilitate recurrent UTIs: the gut, quiescent intracellular reservoirs, and the vagina. In the latter, bacteria may coexist with commensals and adhere/invade the vaginal epithelial cells (Brannon et al. 2020). While this has been well documented for pathogens like *Escherichia coli*, the role of the vagina as a reservoir for recurrent enterococcal UTIs remains to be confirmed. Our group has begun to address this question by examining the surface invasive capacity of *E. faecalis* strains isolated from patients with recurrent UTIs. We observed that vaginal isolates from patients experiencing recurrent UTIs demonstrated enhanced surface penetration *in vitro* compared to urine isolates or laboratory strains, suggesting a potential link between *E. faecalis* with enhanced surface invasiveness and a predisposition for persistent UTIs (Sansone et al. 2024). Further studies employing vaginal epithelial tissues and *in vivo* models are needed to determine whether the colonization site influences the invasiveness of these enterococcal strains.

Outstanding questions

Although enterococci typically exist as commensal microbes in the intestine, they can transition into pathogens that infect multiple body sites. Key questions remain about their tissue tropism, interactions with microbial neighbors, pathobiont mechanisms, and evasion of host immunity. Given the differences in mucus thickness and immune cell populations between the small and large intestines, the influence of these factors on enterococcal colonization and persistence is unclear. Furthermore, the spread of *E. faecalis* and other enterococcal species from MLNs to organs like the liver and spleen requires further study. Additionally, attention has turned to other understudied body sites, such as the brain, a site where enterococci seem to reach via the vagus nerve after leaving the GIT. The presence of bacteria in the brain correlates with microglial activation, a marker of neuroinflammation, and with neural protein aggregation, a hallmark of several neurodegenerative diseases (Thapa et al. 2023). Moreover, the impact of enterococci on the oral microbiota, their transmission routes within the oral cavity, and their effects on immune cells beyond macrophages and osteoblasts need further exploration.

Future research should focus on how enterococci evade host defenses, modulate immune responses, and establish persistent infections in the GIT and extraintestinal sites. This includes investigating the specific molecular signals or receptors on the host that facilitate tissue-specific colonization, determining the roles of different immune cell populations in controlling or promoting enterococcal colonization and expansion in various body sites, and establishing whether host or bacterial responses are specific to the colonization site. Additionally, evolutionary studies are needed to determine whether enterococcal strains from dysbiotic guts diverge into lineages distinct from those found at infection sites or in the intestines of healthy hosts and whether evolutionary changes dictate intestinal translocation capability, immune evasion, and spread in those strains. Since specific clonal complexes within *E. faecalis* and *E. faecium* are often linked to infections, multidrug resistance, and hospital persistence (reviewed by Palmer et al. 2014, Monteiro Marques et al. 2023), it is crucial to identify whether these lineages and their pathogenicity factors (e.g. cytolysin) are particularly prevalent in the dysbiotic GIT or enriched at certain host sites.

A deeper understanding of the molecular interactions governing these processes, and the genetic adaptability of enterococci will help clarify how these bacteria interact with host tissues after disseminating from the GIT. This knowledge would provide valuable insights into effective preventive and therapeutic strategies against enterococcal infections.

Acknowledgments

We apologize to colleagues whose work was not cited in this review due to space limitations. Figures were created with BioRender.com.

Conflict of interest: None declared.

Funding

Research in the Morales laboratory has been supported by Weill Cornell Medicine Department of OB/GYN internal funds, Department of Defense grants OC190443 and OC200224 (J.R.C.R.; D.K.M.), the Frueauff Foundation Grant, the Anna-Maria and Stephen Kellen Junior Faculty Fellowship (D.K.M.), the June Allyson Memorial Fund research award grant (D.K.M.), and the Thomas Caputo Research Award (D.K.M.).

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Received 2 May 2024; **revised** 5 August 2024; **accepted** 6 September 2024